Magnolol Affects Cellular Growth and Proliferation in Human Prostate Cancer Cells *In Vitro*

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Abstract

Prostate cancer is the most commonly diagnosed form of cancer in men in Canada and the United States. Both genetic and environmental factors contribute to development and progression of many cancers, including prostate cancer, with prostate cancer affecting many men worldwide. As such there have been many studies into the effects of natural products on this form of cancer. This work demonstrates the effects of magnolol, a compound found in the roots and bark of the magnolia tree *Magnolia officinalis*, on the behavior of LNCaP, DU145 and PC3 human prostate cancer cells *in vitro*. Magnolol was found to be cytotoxic to human prostate cancer cells, and to affect cell cycle progression of DU145 and PC3 cells, resulting in alterations to the cell cycle and subsequently decreasing the proportion of cells entering the G_2/M-phase of the cell cycle. Magnolol inhibited the expression of cell cycle regulatory proteins including cyclins A, B1, D1 and E, as well as CDK2 and CDK4. Protein expression levels of pRBp107 decreased, while pRBp130 and p27 protein expression levels increased in response to magnolol exposure. PC3 cells exposed to magnolol exhibited decreased protein expression of ornithine decarboxylase, a key regulator in polyamine biosynthesis, as well as affecting expression of other proteins involved in polyamine biosynthesis and catabolism. Furthermore, protein expression of the R2 subunit of ribonucleotide reductase was significantly decreased. Cellular signaling pathways were also assayed to determine which, if any, of these pathways magnolol exposure would alter. Finally, magnolol was found to affect the expression of IGF-I and associated proteins in LNCaP and PC3 cells. In both cell lines,

protein expression of IGF-I and IGFBP-5 were significantly decreased while protein expression of IGFBP-3 was significantly increased. The results obtained throughout this thesis suggest that magnolol can affect the behavior of human prostate cancer cells *in vitro*, suggesting that magnolol may have potential as a novel anti-prostate cancer agent.

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List of Abbreviations

α-MEM	Alpha-minimum Essential Medium
Akt	Protein Kinase B
ANOVA	Analysis of Variance
AP	Alkaline Phosphatase
AP-1	Activator Protein-1
ATP	Adenosine-5'-Triphosphate
AZ	Antizyme
AZI	Antizyme Inhibitor
BCE	Before Current Era
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	Bovine Serum Albumin
CDK	Cyclin-dependent Protein Kinase
Cip/Kip	CDK Interacting Protein/Kinase Inhibitory Protein
Ctrl	Control
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DU145	Duke University Prostate Cancer Cell Line 145
ERK	Extracellular Signal-regulated Kinase
FBS	Fetal Bovine Serum
G	Gravity

GH	Growth Hormone
GPCR	G Protein-coupled Receptor
GSK3β	Glycogen Synthase Kinase-3-beta
h	Hour(s)
HPLC	High-performance Liquid Chromatography
IκB	Inhibitor of κB
IκBα	Inhibitor of κB Alpha
IGF	Insulin-like Growth Factor
IGF-I	Insulin-like Growth Factor-1
IGF-II	Insulin-like Growth Factor-2
IGF-IIR	Insulin-like Growth Factor-2 Receptor
IGF-IR	Insulin-like Growth Factor-1 Receptor
IGF-R	Insulin-like Growth Factor Receptor
IGFBP	Insulin-like Growth Factor Binding Protein
IKK	IκB Kinase
INK4	Inhibitor of Kinase 4
IRS	Insulin Receptor Substrate
JNK	c-Jun N-terminal Kinase
LNCaP	Lymph Node Carcinoma of the Prostate
MAP2K	Mitogen-activated Protein Kinase Kinase
MAP3K	Mitogen-activated Protein Kinase Kinase Kinase
MAPK	Mitogen-activated Protein Kinase

MEK	Mitogen-activated Protein Kinase/Extracellular Signal-regulated Kinase Kinase
min	Minute(s)
MKK	Mitogen-activated Protein Kinase Kinase
MMP	Matrix Metalloproteinase
mTOR	Mammalian Target of Rapamycin
NBT	Nitro Blue Tetrazolium
NFκB	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
NSILA	Non-suppressible Insulin-like Activity
ODC	Ornithine Decarboxylase
p-	Phosphorylated-
PAGE	Polyacrylamide Gel Electrophoresis
PAO	Polyamine Oxidase
PBS	Phosphate Buffered Saline
PC3	Prostate Cancer Cell Line 3
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-4,5-biphosphate
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PMSF	Phenylmethylsulfonyl Fluoride
PTEN	Phosphatase and Tensin Homologue
RB	Retinoblastoma
Rel	Reticuloendotheliosis

RHD	Rel Homology Domain
RNA	Ribonucleic Acid
RNAse	Ribonuclease
RPMI	Roswell Park Memorial Institute Medium
RTK	Receptor Tyrosine Kinase
SAMDC	S-Adenosylmethionine Decarboxylase
SAPK	Stress Activated Protein Kinase
SDS	Sodium Dodecyl Sulfate
sec	Second(s)
SSAT	Spermidine/Spermine N^1-Acetyltransferase
TBS	Tris-buffered Saline
TRAMP	Transgenic Adenocarcinoma of the Mouse Prostate
Tris-HCl	Tris(hydroxymethyl)aminomethane Hydrochloride
WCE	Whole Cranberry Extract

Epigraph

"Tiger got to hunt, bird got to fly;

Man got to sit and wonder 'why, why, why?'

Tiger got to sleep, bird got to land;

Man got to tell himself he understand."

- Kurt Vonnegut, *Cat's Cradle*

CHAPTER ONE

Introduction and Literature Review

1.1 Introduction

Prostate cancer is the most common form of cancer among men in Canada and the United States, and the second most common form of cancer among men worldwide (ASC 2011; CCS 2013). The complex nature of the disease combined with the difficulties in finding effective treatments with few side effects have led to a consistent search for new compounds with chemopreventative and chemoprotective properties. One area of interest is the search for novel, naturally occurring, anti-cancer compounds. Many herbal and phytochemical compounds have been shown to have some chemopreventative effect, and there is evidence to suggest that a relationship between these compounds in the diet and incidence of cancer exists (Aggarwal and Shishodia 2006; Bemis *et al*. 2006; Neto 2011; Stein and Colditz 2004; Tan *et al*. 2011). One such natural product is magnolol, a compound found in the roots and bark of the magnolia tree *Magnolia officinalis*. Magnolol has previously been shown to induce apoptosis and to decrease metastasis in human prostate cancer cells *in vitro*, as well as having a variety of other anti-cancer effects on various other types of cancer *in vitro* (Hwang *et al*. 2013; Lee *et al*. 2009). The work presented seeks to further demonstrate the anti-cancer effects of magnolol in human prostate cancer cells *in vitro* by investigating the effect of magnolol on cellular growth and proliferation lined activities within the cell.

1.1.1 Research Rationale, General Hypothesis and Objectives

Previous research on the effects of magnolol on various human cancer cell lines has been conducted. Magnolol is known to inhibit metastasis in human ovarian cancer cells *in vitro*, and to induce apoptosis in human gastric cancer cells *in vitro* (Chuang *et al*. 2011; Rasul *et al*. 2012). Furthermore, magnolol has been reported to suppress proliferation and activate apoptosis in human colon and liver cancer cells *in vitro* and *in vivo*, as well as cause cell cycle arrest in human colon cancer cells *in vitro* (Hsu *et al*. 2007; Lin *et al*. 2001; Lin *et al*. 2002). Finally, magnolol has been shown to inhibit cellular proliferation and induce apoptosis in glioblastoma cells *in vitro* and to cause cell cycle arrest in human urinary bladder cancer cells *in vitro* (Chen *et al*. 2009; Chen and Lee 2013; Lee *et al*. 2008a).

Specific to prostate cancer, magnolol has previously been shown to induce apoptosis and to suppress metastasis in human prostate cancer cells *in vitro* (Hwang and Park 2010; Lee *et al*. 2009). The precise effects of magnolol on cellular growth and proliferation in human prostate cancer cells have not, however, been examined. The purpose of this work is to expand the current understanding of magnolol as a potential anti-prostate cancer compound by focusing on the magnolol-mediated cellular response with specific regard to cellular growth and proliferation. It was therefore hypothesized that **magnolol will inhibit cancer cell growth *in vitro***, specifically that magnolol will affect human prostate cancer cells *in vitro* by affecting activities linked to cellular growth and proliferation.

The specific objectives of this thesis are:

1) Determination of the effect of magnolol on cell cycle events.
2) Determination of the effect of magnolol on cellular proliferation-linked activities.
3) Determination of the effect of magnolol on insulin-like growth factor-I and associated proteins.

Each of the three objectives outlined above was achieved, and have been individually submitted for publication as research manuscripts. All three of those manuscripts have been published in academic journals. As such, these objectives have been organized as three separate manuscripts which comprise Chapters Two, Three and Four of this thesis. Together, these chapters represent the knowledge/discovery section of this publication-style thesis.

1.2 Literature Review

1.2.1 Cancer

Cancer has been known to medicine since ancient times. The earliest evidence of cancer can be found in Egyptian manuscripts dating back to 1600 BCE (Sudhakar 2009). In these manuscripts it was noted that the appropriate treatment for surface tumors was surgical removal or cauterization, while deep tissue tumors were considered untreatable (Sudhakar 2009). The word cancer first appears as the Greek *karkinos* described by Hippocrates (460-370 BCE) (Sudhakar 2009). Hippocrates is considered the first Greek physician to have studied the specifics of cancer but, as with the ancient Egyptians, considered deep or "hidden" cancers to be incurable as the patients often lived longer if left untreated (Karpozilos and Pavlidis 2004). Later Greek physicians, such as Galen (129-199), determined that there was a connection between diet, environment and cancer occurrence (Karpozilos and Pavilidis 2004). Despite these discoveries, the treatment of cancer remained essentially unchanged from the methods of the ancient Egyptians throughout the middle ages.

Today, cancer is considered the fastest growing disease worldwide with regard to new diagnoses and deaths and is becoming the leading cause of death in developed countries like Canada, where cancer is the leading cause of death, and the United States of America where it is the second leading cause of death (ACS 2011, 2014; CCS 2013). In developing countries cancer is second only to heart disease as the leading cause of death (Popat *et al*. 2013). Lung cancer is the leading cause of new cancer cases in men while breast cancer is the leading cause in women worldwide (ACS 2011). In developed

countries prostate cancer is the leading cause in men while breast cancer remains the leading cause in women (ASC 2011). In 2008, 12.7 million new cases of cancer were estimated to have occurred worldwide and by 2030 it is estimated that there will be 21.4 million new cases of cancer per year (ACS 2011). In 2013, an estimated 187600 Canadians developed cancer and 75500 Canadians are estimated to have died of cancer (CCS 2013). It is estimated that 2 in 5 Canadians will develop cancer in their lifetime and that 1 in 4 Canadians will die of cancer (CCS 2013). Lung, colorectal, breast and prostate cancer each account for 10% or more of all cancer death in each sex in Canada and more than half of all new cases will be one of these types of cancer (CCS 2013).

Increasing cancer rates have been linked to an aging population, with 78% of all new diagnoses occurring after age 55 worldwide, and 88% of all diagnoses in Canada occurring after age 50 (ACS 2011; CCS 2013). Within Canada there are higher rates of cancer on the east coast, with rates gradually decreasing westward (CCS 2013). Other factors contributing to increased risk of cancer are: tobacco use, physical activity, weight, diet, alcohol use and sun exposure (Stein and Colditz 2004). Of particular concern is the association between obesity and cancer, with roughly 25% of Canadian adults considered obese according to measurements taken from 2007-2009 and increasing rates of obesity in both adults and children in both developed and developing countries (PHAC 2011; WHO 2000). As rates of cancer and obesity both increase the relation between the two is of increasing importance as diet and body weight are the two most common causes of cancer in the United States (Wolin *et al*. 2010). Obesity has been linked to increased activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-

kinase (PI3K) cellular signaling pathways as well as increased expression of insulin-like growth factor-I (IGF-I) (Vucenik and Stains 2012). These processes are all associated with cellular proliferation and increases in expression of proteins involved in these pathways would lead to an increased propensity for cancer cell development.

Cancer is typically recognized as a group of diseases representing the uncontrolled growth and spread of cells (ACS 2014). While normal cellular proliferation follows a strictly controlled sequence of growth, division and death, cancer cells undergo growth and proliferative processes in an unregulated manner and avoid apoptosis. Hanahan and Weinberg (2000, 2011) have listed six hallmark characteristics of cancer cells. Those characteristics which all forms of cancer share are: sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting cell death. This list was also updated to include two additional emerging hallmarks: deregulating cellular energetics and avoiding immune destruction; and two enabling characteristics: genome instability/ mutation and tumor-promoting inflammation (Hanahan and Weinberg 2011). These hallmarks and enabling characteristics represent the key processes involved in cancer treatment and cancer research as compounds which disrupt these hallmarks would represent a potentially novel compound for cancer treatment. As all of these hallmarks appear necessary for cancer development, cancer is a complex and widely diverse disease with each of these characteristics developing independently and in different ways. As this thesis is primarily concerned with cellular proliferation it is important to note that Hanahan and Weinberg (2011) recognized the hallmark of sustaining proliferative

signaling as “arguably the most fundamental trait” in cancer development. Those pathways proposed as most commonly activated in carcinogenesis are the MAPK and PI3K/Akt signaling pathways (Hanahan and Weinberg 2011). In their discussion of the “evading growth suppressors” hallmark Hanahan and Weinberg (2011) found the RB growth suppressor proteins as the most typically downregulated. This also means that as cells develop those traits needed for cancer development there is a selection whereby cells which are growing faster, dividing more often and resisting cell death longer will be selected for. This allows for further characteristics of cancerous cells to develop simply because these cells are behaving in ways that non-cancerous cells do not. This kind of selection of has been linked to genetic instability as cells exhibiting an increased propensity for genetic instability, and thereby gaining the hallmarks of cancer through DNA damage, acquire cancerous traits and retain their instability which allows for the possibility of acquiring further traits (Cahill *et al*. 1999).

Treatment of cancer today includes such methods as surgical intervention, radiation therapy, chemotherapy, hormone therapy and monitoring (DeSantis *et al*. 2014). One challenge in developing new treatments for cancer is in targeting those treatments to specific tumor types, this kind of targeting leads to maximized efficacy with minimal toxicity to non-cancerous cells (Golub *et al*. 1999).

1.2.1.1 Prostate Cancer

The prostate gland is an accessory organ involved in male reproduction. The prostate gland is composed of both epithelial and stromal cells and it is the epithelial cells which typically prove cancerous as these cells lose the need for support by the stromal cells (Russell *et al*. 1998). Prostate cancer is the second most common cancer in men worldwide and the most common cancer in men in Canada (ACS 2011; CCS 2013). It is estimated that 24.5% of all new cancer diagnoses in men in 2013 were prostate cancer and that Canadian men have a 1 in 7 chance of developing prostate cancer in their lifetime (CCS 2013). As with other forms of cancer, the probability of prostate cancer diagnosis increases with age. Prostate cancer is rarely diagnosed in men under 50 years, with the majority of diagnoses occurring between the ages of 55 and 64 years worldwide (Baade *et al*. 2009; Dunn and Kazer 2011). Traditionally, those diagnosed with prostate cancer at a younger age present with more aggressive disease and poorer overall prognosis but as testing methods develop and improve there has been a decrease in age at diagnosis and that disease is less severe at diagnosis due to early detection (Baade *et al*. 2009).

Risk factors for prostate cancer include: family history, advanced age, race, diet and previous vasectomy (Dunn and Kazer 2011; Pienta and Esper 1993). It is of particular interest that prostate cancer has been associated with a Western lifestyle, with lower rates of prostate cancer occurring in Asian countries (Grönberg 2003; Hsing and Chokkalingam 2006). The connection between the increased rate of prostate cancer in Western countries appears to be largely due to diet, with increased red meat consumption,

increased availability of androgens and estrogens, and increased dietary fat intake (Grönberg 2003; Hsing and Chokkalingam 2006). This is further supported by the lower incidence of prostate cancer in populations with an Asian or vegetarian diet (Grönberg 2003; Hsing and Chokkalingam 2006). It has also been suggested by Pienta and Esper (1993) that socioeconomic status and occupation may also play a role in prostate cancer risk, but these factors have not been conclusively linked to the disease.

After diagnosis there are several treatment options available for prostate cancer, the most common of which include: active surveillance, prostatectomy, external radiation therapy, brachytherapy, cryotherapy, hormone therapy and chemotherapy (Dunn and Kazer 2011). Active surveillance is most commonly used after initial diagnosis for early stage prostate cancers or in individuals who would otherwise have a life expectancy of under 10 years (Dunn and Kazer 2011). This allows for monitoring of disease advancement while maintaining the highest quality of life for as long as possible as other treatment options are more extreme in nature. Chemotherapy is generally used as the last option for advanced, metastatic disease (Dunn and Kazer 2011).

As noted above, treatment of prostate cancer is usually based upon disease progression. Androgens are important for the growth, development and proliferation of normal prostate cells as well as for early stages of prostate cancer. Prostate cancer tumor growth is initially androgen dependent, with cancer cells requiring the presence of androgens to divide as would normal prostate cells (Feldman and Feldman 2001; Galbraith and Duchesne 1997). At this stage androgen ablation, through either surgical or chemical deprivation of androgens, is effective at slowing or causing regression of

prostate cancer tumor growth (Feldman and Feldman 2001). In normal prostate cells and in androgen dependent prostate cancers the loss of androgens will result in slowed cellular proliferation and increased apoptosis (Galbraith and Duchesne 1997). Generally the regression of tumor growth resulting from androgen ablation is a temporary solution, with the development of androgen independent prostate cancer occurring eventually. Prostate cancer typically progresses by changing from androgen dependence, where androgens are required for cellular growth and division, to androgen sensitivity, where cells will grow and divide faster in the presence of androgens but those androgens are not required for cellular growth, to full androgen independence (Russell *et al*. 1998). This kind of progression will happen almost invariably after androgen ablation therapy, typically taking about 24 months for androgen independence to fully develop after treatment (Russell *et al*. 1998). It has been posited that all metastatic prostate cancers will eventually become androgen independent (Galbraith and Duchesne 1997). This development occurs because androgen dependent prostate cancer cells are being selected against due to the loss or limitation of androgens, allowing androgen independent cells to gain a selective growth advantage. Androgen independence can be acquired though several different kinds of genetic mutation which may allow androgen receptors to stimulate growth in the absence of androgen, allow receptors to become more sensitve to low levels of androgens or to become sensitive to non-androgen steroids hormones (Feldman and Feldman 2001; Galbraith and Duchesne 1997). These androgen independent cells represent a much more aggressive form of prostate cancer and it is at

this stage that chemotherapy is normally used in disease treatment (Dunn and Kazer 2011).

As the aggressiveness of disease increases so too does the rate of metastasis, with bone being the most common site of metastatic invasion (Dunn and Kazer 2011). It was found that 84% of metastatic prostate cancers in a US population between 1998 and 2010 showed metastasis to bone, with the next most common site being lymph nodes at 10.6% (Gandaglia *et al*. 2014). The basis of this preference for bone metastasis is not well understood but several theories are being investigated. The most popular hypotheses concern bone presenting a microenvironment closely matching the needs of prostate cancer cells (Gandaglia *et al*. 2014; Rucci and Angelucci 2014). Rucci and Angelucci (2014) further suggest that growth factors stored in the bone matrix become available to prostate cancer cells and so stimulate proliferation. This hypothesis is further expanded by Autio and Morris (2013) who suggest that the ionized calcium released by osteoclast activity stimulates proliferation as well as encouraging cells to migrate to bone. Finally, the veins between the prostate and lower vertebrae may provide direct access for prostate cancer to invade the spinal cord (Gandaglia *et al*. 2014; Rucci and Angelucci 2014).

1.2.1.2 *In Vivo* Models of Prostate Cancer

For research on prostate cancer to progress, a model of the disease is necessary for researchers to work with. Ideally, this model would closely mimic clinical conditions of human prostate cancer such as occurring spontaneously, having a reasonably slow doubling time, be androgen dependent or sensitive with the ability to progress to androgen independence, produce prostate specific antigen and commonly metastasize to bone and lymph nodes (Bosland *et al*. 1996; Russell *et al*. 1998). Several different animal models are in use for prostate cancer research, including rat and dog models which are capable of spontaneously developing carcinoma of the prostate, but the tumors developed do not behave as human prostate cancers do and are only useful as models of certain stages of tumor growth and cancer development (Lamb and Zhang 2005).

Genetically engineered mouse models of prostate cancer are also in use, with one of the earliest developed being the transgenic adenocarcinoma of the mouse prostate (TRAMP) model (Lamb and Zhang 2005). TRAMP mice were developed in the early 1990's as a spontaneously occurring model of prostate cancer (Greenberg *et al*. 1995; Valkenburg and Williams 2011). To produce TRAMP mice, a viral oncogene was inserted which is specifically activated in the mouse prostate and leads to the downregulation of p53 and pRB expression (Greenberg *et al*. 1995; Valkenburg and Williams 2011). TRAMP mice have a high rate of tumor incidence and the disease presented mimics the histology and pathology of human disease, making them a popular model in nutrition and chemoprevention studies (Lamb and Zhang 2005). The strength of the TRAMP model is that it represents a spontaneous cancer of the mouse prostate which mimics the

progression of human disease in that it can progress to androgen independence and is metastatic (Valkenburg and Williams 2011). This allows for the *in vivo* study of a cancer in the animal in which it has originally occurred. The weakness of the TRAMP model is that cancers are not of the same epithelial origin as in human disease and metastasis to bone rarely occurs (Valkenburg and Williams 2011).

Finally, immunodeficient mice such as the nude mouse model have been used, where human prostate cancer cells are implanted into these mice (Lamb and Zhang 2005). Nude mice are deficient in T lymphocytes due to the lack of a thymus gland and therefore show no immune response to foreign tissue (Valkenburg and Williams 2011). This allows for human prostate cancer cells to be xenografted into these mice where they can develop into tumors without being destroyed by the mouse immune response. The advantage to using such a xenograft model lies in the ability to study human prostate cancer cells *in vivo*, undergoing angiogenesis, metastasis and hormonal regulation in the context of a whole animal. Using this xenograft model does, however, require the development of human prostate cancer tissue culture cell lines. The disadvantages to using the nude mouse xenograft model is that the compromised immune system of these animals may affect tumor progression and metastasis, causing changes which many not occur in animals with functioning immune response to tumors, and that this model does not adequately represent the interaction of cancerous cells within the microenvironment (Valkenburg and Williams 2011).

The mouse models have various strengths and weaknesses in the context of prostate cancer research. The support for mouse models arises from the ability to study

cancer cells *in vivo*, the fact that the mouse and human genomes share 95% similarity, mice are easily genetically modified, develop quickly and are easy to raise in the large numbers required for study (Valkenburg and Williams 2011). Some problems with the mouse model of prostate cancer do, however, remain. Naturally occurring prostate cancer is uncommon in mice, and the mouse and human prostates are dissimilar in structure (Valkenburg and Williams 2011). Most mouse metastases tend to originate from mesenchymal cells, whereas most human metastases originate from epithelial cells meaning that the mouse models represent a different form of disease (Valkenburg and Williams 2011). Lastly, bone metastasis is very uncommon in mice, even when implanted with human prostate cancer cells, while bone is the most common site of metastasis in humans (Valkenburg and Williams 2011). As no idealized animal model exists, with spontaneous prostate cancer development being rare in animals other than humans, several human prostate cancer tissue culture cell lines have been developed for the study of prostate cancer (Sobal and Sadar 2005).

1.2.1.3 *In Vitro* Models of Prostate Cancer

Due in part to the limitations of producing animal models of prostate cancer (ethical concerns, expense and lack of appropriate model), several human prostate tissue culture cell lines have been developed (Sobal and Sadar 2005). The first such attempts at isolating a human prostate cancer tissue culture were made as early as 1917 but these initial efforts provided only short-term, primary cultures (Burrows *et al*. 1917; van Bokhoven *et al*. 2003). Progress toward a viable long term tissue culture of human prostate cancer was not made until the 1970's, yet these cell lines were susceptible to contamination from other cells in culture and many were found to have been contaminated by other tissue cultures such as HeLa cells (van Bokhoven *et al*. 2003). Prostate cancer has proven to be one of the most difficult cell types from which to establish a continuous cell line because of the various different tissue types found in the prostate (Russell *et al*. 1998; van Bokhoven *et al*. 2003). As such, development of these cell lines often involves metastatic cancers as tumors found in these metastasis represent a homogeneous population comprised only of malignant cancer cells. By developing tissue cultures from specimens taken from these sites of metastasis the issue of having cells representative of both normal prostate and cancerous prostate is avoided (Stone *et al*. 1978). The three "classical" human prostate cancer tissue culture cell lines were developed in exactly this way.

There are three "classical" *in vitro* models of human prostate cancer: LNCaP, DU145 and PC3 cells. These three cell lines remain the most popular models for the majority of prostate cancer research (Sobal and Sadar 2005). As it is necessary for models

to closely represent clinical conditions of human prostate cancer these three cell lines are useful as they present a natural progression of disease when used together (Bosland *et al*. 1996; Nair *et al*. 2004). LNCaP cells represent a poorly malignant and androgen-sensitive prostate cancer, DU145 cells are moderately malignant and androgen-independent and PC3 cells are a highly malignant prostate cancer cell line which is also androgen-independent (Nair *et al*. 2004). These cell lines are recognized as being useful to the study of prostate cancer and have previously been used to justify clinical trials (Lippman *et al*. 2009). A study by van Bokhoven *et al*. (2003) showed that the karyotypes of these "classical" cell lines remained similar to those originally reported and determined that these *in vitro* models of prostate cancer retained a high level of stability over time. It is important, however, to keep in mind the number of times a tissue culture cell line has been subcultured to ensure that experimental results are as accurate and repeatable as possible. Passage number refers to the number of times a tissue culture cell line has been transferred from one vessel to another, or subcultured, for the purpose of propagating cells and maintaining tissue culture health (ATCC 2010). Researchers are becoming increasingly aware that the characteristics of cell lines can alter as high passage numbers are reached (ATCC 2010). High passage numbers can lead to changes in morphology, response to experimental compounds and growth rates so it is important to avoid using cells which have been subcultured too many times (ATCC 2010). This can be achieved by cryopreserving cells at low passage numbers and frequently replacing tissue cultures with these preserved low passage cells as the passage number of cultures increases. PC3 cells, representing the most advanced disease state of the three models, have been chosen as the

primary tissue culture to be studied in this thesis with supporting data from DU145 and LNCaP cells included in selected studies. The decision to focus on PC3 cells, with select supporting data from other cell lines, is consistent with previous *in vitro* studies on human prostate cancer cells (Lee *et al*. 2009).

LNCaP cells were isolated from a lymph node metastasis of prostate cancer in a 50 year old, white male (Horoszewicz *et al*. 1980). Unlike previously established cell lines, LNCaP cells were found to be weakly adherent and much slower growing with a doubling time of approximately 72 h (Horoszewicz *et al*. 1980). This cell line also retained its malignant properties when implanted into nude mice after cell culture but would establish much faster in male mice than in females (Horoszewicz *et al*. 1980, 1983). LNCaP cells were found to be capable of growth in the absence of androgens but to grow much faster when androgens were present, establishing that LNCaP cells were androgen-sensitive (Horoszewicz *et al*. 1980, 1983).

DU145 cells were isolated from a brain metastasis of prostate cancer in a 69 year old, white male (Stone *et al*. 1978). These cells were described as being a poorly differentiated adenocarcinoma which changed little in 2 years of subsequent tissue culture showing a doubling time of approximately 34 h (Stone *et al*. 1978). The cells were noted as androgen-independent as cells would grow equally well in either the presence or absence of androgens (Stone *et al*. 1978).

PC3 cells were isolated from a lumbar vertebrae metastasis of prostate cancer in a 62 year old, white male (Kaighn *et al*. 1979). These cells were described as having good adhesion to tissue culture dishes and showing reliable growth with a doubling time of

approximately 33 h (Kaighn *et al*. 1979). It was noted that no growth factors stimulated the growth of PC3 cells, that cells required minimal serum and that the addition of dihydrotestosterone did not affect cellular growth; this androgen-independence was confirmed in that tumors readily developed when implanted in either male or female nude mice (Kaighn *et al*. 1979). This line has subsequently been recognized as the most aggressive of the "classical" prostate cancer tissue cultures (Sobal and Sadar 2005).

1.2.2 The Magnolia Tree, *Magnolia officinalis* and Magnolol

The magnolia tree *Magnolia officinalis* is widely used in traditional Chinese medicine (Figure 1.1 A). These trees are found throughout subtropical China at 300-2000 m elevation, typically in broad leaf forests (He *et al*. 2009). *Magnolia officinalis* trees typically have a life span of over 100 years and do not bear fruit until approximately 15 years of age (He *et al*. 2009). The roots and bark of *Magnolia officinalis*, known as the herb Hou pu, have traditionally been used to treat a variety of symptoms and complaints such as gastrointestinal disorders, anxiety and allergies (Lee *et al*. 2011). More recently, extracts from magnolia bark have shown anti-cancer, anti-inflammatory, anti-oxidant, anti-depressant, anti-Alzheimer's and anti-atherosclerosis effects (Lee *et al*. 2011).

This bark was found to contain several biologically active compounds including honokiol, 4-*O*-methylhonokiol, obovatol and magnolol with magnolol being the most abundant (Lee *et al*. 2011). Magnolol is a phenolic compound with the chemical structure 5,5'-diallyl-2,2'-dihydroxylbiphenyl (Figure 1.1 B) (Bang *et al*. 2000). Compounds like magnolol are gaining increased attention due to their therapeutic potential and historical usage (Chen *et al*. 2011; Mainardi *et al*. 2009). As increasing research on magnolol is being undertaken it has been found that magnolol can exhibit anti-microbial activity at a concentration of 25 μg/ml and anti-fungal activity at a concentration of 25-100 μg/ml (Bang *et al*. 2000; Ho *et al*. 2001). Magnolol has also been found to display a variety of medicinal effects originally attributed to magnolia bark, as well as being widely bioavailable (Chen *et al*. 2011). These attributes make magnolol an important candidate for research into bioactive natural compounds.

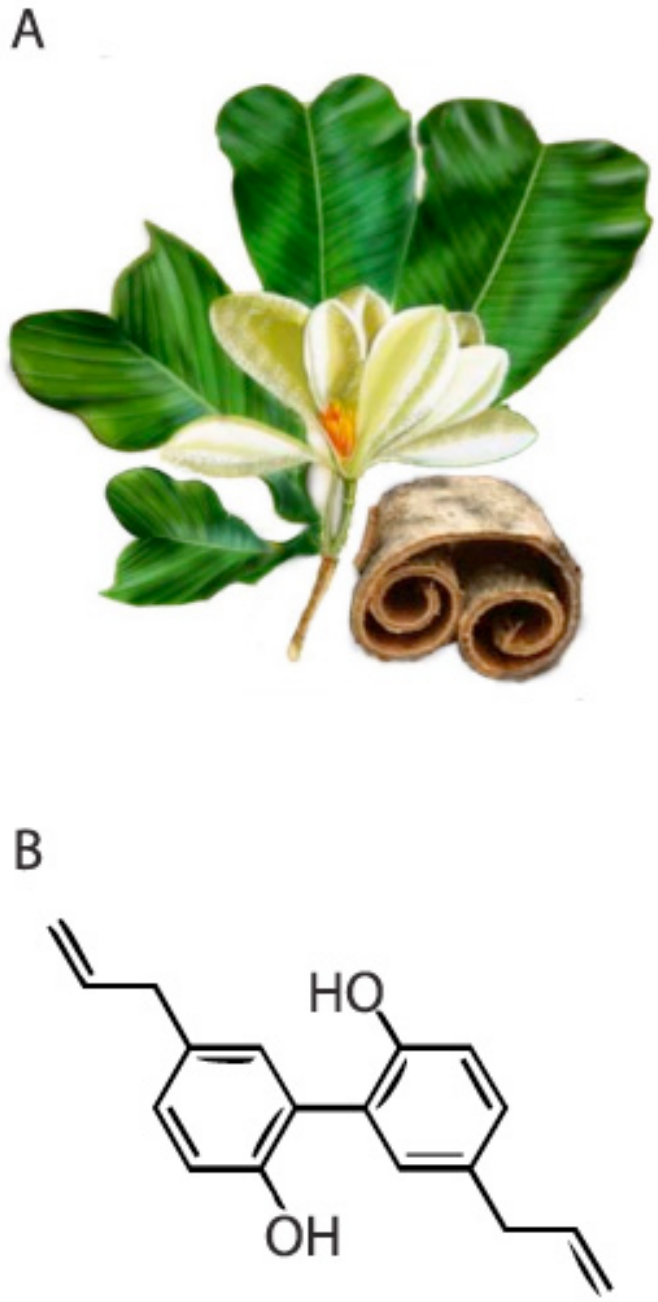

Figure 1.1: *Magnolia officinalis* and Magnolol. (A) The leaf, flower and bark of the magnolia tree *Magnolia officinalis* (Adapted from Chen *et al*. 2011). (B) The chemical structure of magnolol, 5,5'-diallyl-2,2'-dihydroxylbiphenyl ($C_{18}H_{18}O_2$).

1.2.2.1 Effects of Magnolol on Cancer

Of particular interest are the anti-cancer and anti-prostate cancer effects attributed to magnolol exposure. A variety of studies have been done on magnolol and cancer and they show that this compound is capable of inducing apoptosis and preventing both metastasis and cellular growth and proliferation. Magnolol has previously been shown to induce apoptosis in a variety of human cancer cell lines, both *in vitro* and in cancerous cells implanted into animal models. Apoptosis was induced in HepG2 human hepatoma and COLO205 human colon cancer cell lines, both *in vitro* and *in vivo* (Lin *et al*. 2001, 2002). Apoptosis induction also occurred in human gastric cancer cells, glioblastoma cells and breast cancer cells exposed to magnolol (Chen and Lee 2013; Rasul *et al*. 2012; Zhou *et al*. 2013). With regard to metastasis, magnolol has been shown to reduce metastatic potential in human ovarian cancer cells (Chuang *et al*. 2011).

Magnolol has also previously been shown to affect cellular growth and proliferation in many different human cancer cell lines *in vitro*. This anti-proliferative effect is generally measured though cell cycle arrest and accompanied by increased expression of either p21 or p27, two cyclin dependent kinase inhibitors. Cell cycle arrest occurred at different stages of the cell cycle across different cell lines. Human gastric cancer cells were arrested in the S phase of the cell cycle (Rasul *et al*. 2012). Human breast cancer cells and human urinary bladder cancer cells were both arrested in the G2/M phase of the cell cycle (Lee *et al*. 2008a; Zhou *et al*. 2013). Finally, human glioblastoma cells, human leukemia cells and human epidermal carcinoma cells were all arrested in the G0/G1 phase of the cell cycle (Chen *et al*. 2009; Chilampalli *et al*. 2011;

Fong *et al*. 2005). Of those studies reporting an increase in either p21 or p27, p21 increased in human colon cancer cells, glioblastoma cells and epidermal carcinoma cells while p27 increased in human urinary bladder cancer cells and leukemia cells (Chen *et al*. 2009; Chilampalli *et al*. 2011; Fong *et al*. 2005; Hsu *et al*. 2007; Lee *et al*. 2008a).

At the time of writing only five papers are known to have been published regarding the effects of magnolol on prostate cancer, three of which comprise Chapters Two, Three and Four of this thesis. Of the remaining two papers, one focuses on the effects of magnolol on apoptosis in human prostate cancer cells while the other explores the anti-metastatic effects of magnolol on human prostate cancer cells. The apoptosis study focuses on the PC3 cell line, but includes cytotoxicity data from DU145 and LNCaP cells as well. Magnolol was found to be cytotoxic to all three cancerous prostate cell lines tested and induced apoptosis in PC3 human prostate cancer cells *in vitro* at a concentration of 60 μM magnolol after 8 h (Lee *et al*. 2009). Cellular signaling pathways were then tested to determine the mechanisms by which magnolol affected these cells and it was found that magnolol caused an increase in caspase-3 expression and decrease in p-Akt and p-PI3K expression (Lee *et al*. 2009). This suggests that magnolol signals through the PI3K/Akt pathway in PC3 human prostate cancer cells. The metastasis study also focused on PC3 human prostate cancer cells exposed to 0-40 μM magnolol for 60 min to 24 h time intervals (Hwang *et al.* 2010). It was found that magnolol decreased cellular proliferation of PC3 cells, as well as cellular adhesion and invasion in a dose-dependent manner (Hwang *et al*. 2010). Protein expression of the matrix metalloproteinases (MMP), specifically MMP-2 and MMP-9, were significantly reduced and wound-healing was

delayed (Hwang *et al*. 2010). This metastasis study showed that magnolol is capable of slowing the spread of PC3 human prostate cancer cells *in vitro* but makes note that the specific mechanisms by which this occurs remain unknown.

1.2.2.2 Bioavailability, Delivery and Toxicity of Magnolol

When investigating a compound for its biological effects *in vitro* it is important to keep in mind the bioavailability and toxicity *in vivo* to ensure that concentrations investigated are appropriate and meaningful. In an overview of magnolol as a compound important to Chinese traditional medicine Chen *et al*. (2011) noted that magnolol has a "desirable spectrum of bioavailability" in that the compound is readily absorbed and is capable of crossing the blood-brain barrier. It is also noted that magnolol has historically been used at very high concentrations and for long term administration periods and toxicity has not been observed to occur (Chen *et al*. 2011).

Bioavailability studies performed in mouse and rat models have shown that, using two different methods of delivery, substantial concentrations of magnolol in the blood plasma could be achieved. Intravenous injection and oral administration have previously proven to be the most effective methods of delivery, with an injected dose of 2-10 mg/kg attaining a maximum blood plasma concentration of 10-40 μM magnolol while oral administration of 20 mg/kg could attain a maximum blood plasma concentration of 0.1-0.4 μM magnolol (Ho and Hong 2012; Lin *et al*. 2011; Tsai *et al*. 1996). A third method of delivery, topical application, has been examined but this study found that while some absorption of magnolol into the skin occurred the concentrations would not likely be pharmacologically active with the maximal concentration achieved being 0.22 nmol/mg (Lin *et al*. 2013). It was found that the elimination half-life for magnolol was 15 min, with total body clearance at 72-75 ml/min/kg (Ho and Hong 2012; Homma *et al*. 1993; Lin *et al*. 2011). After oral treatment magnolol has been observed to be retained in

the blood plasma for up to 1 h, with total recovery of magnolol in the urine being 16.7% of that administered (Ho and Hong 2012; Homma *et al*. 1993).

In the study regarding the effects of magnolol on apoptosis in human prostate cancer cells mentioned above, both cancerous and non-cancerous cells were exposed to magnolol. While magnolol proved cytotoxic to the LNCaP, DU145 and PC3 human prostate cancer cell lines, non cancerous cells assayed showed little cytotoxicity *in vitro* (Lee *et al*. 2009). These non-cancerous cell lines included rat prostate endothelial cells (YPEN-1), immortalized nonmalignant human breast epithelial (MCF-10A) and a primary culture of human prostate epithelial cells (PrECs); these PrECs in particular showed the least cytotoxicity of all cells tested (Lee *et al*. 2009). Magnolol was also shown not to be mutagenic, and in fact to have anti-mutagenic effects, both *in vitro* and *in vivo* in a study which deemed magnolia bark extract safe for human dietary consumption (Lee *et al*. 2011; Li *et al*. 2007). Pre-clinical *in vivo* studies on the toxicity of magnolol have also been performed. These studies have shown that magnolol was non-toxic at every tested dose and that there were no drug related side effects nor alterations in immune response associated with magnolol exposure (Ho and Hong 2012; Li *et al*. 2007; Liu *et al*. 2007). The highest tested concentrations of these experiments were: 2.5 g/kg in mice, 0.48 g/kg/day for 21 days in rats and 0.24 g/kg/day for 90 days in rats (Ho and Hong 2012; Li *et al*. 2007; Liu *et al*. 2007). There was no meaningful change in kidney or thyroid weight, no dose-related difference in body weight or weight gain in any of the rats treated with magnolol and no meaningful change in blood or urine chemistry in any rats exposed to the highest tested dose of magnolol in these studies (Liu *et al*. 2007).

1.2.3 The Cell Cycle

For cells to multiply they must undergo a process of cellular division whereby the genetic material of the cell is duplicated, the cells grow in size and the cells divide into two identical daughter cells. For this kind of mitotic cellular division to occur cells must transition through the cell cycle, where each stage of the process is controlled. Cell division was originally divided into two different phases: mitosis (M) and interphase. This separation of cell division into two distinct phases was based on what was visibly happening in the cell, under a light microscope, during division: during mitosis chromosomes could be seen condensing, aligning themselves and separating, while during interphase the cells merely seemed to increase in size (Nasmyth 1996; Vermeulen *et al*. 2003).

The M phase is easily broken down based on what was visually occurring: in prophase the chromosomes become visible, in metaphase chromosomes align themselves across the middle of the cell, in anaphase the chromosomes separate to either pole of the cell, and in telophase chromosomes uncoil and are no longer visible. Upon the discovery that DNA synthesis occurred during a restricted portion of the interphase more investigation into interphase itself was warranted (Howard and Pelc 1951). The interphase was subsequently determined to consist of three different steps (Figure 1.2): G_1, the first gap after mitosis where cells prepare for DNA synthesis; S, where DNA synthesis occurs; and G_2, where cells prepare for mitosis (Nasmyth 1996; Vermeulen *et al*. 2003). Cells in G_1 can also enter G_0 before committing to DNA replication, where G_0

is a resting state representing cells which are not actively growing or dividing (Vermeulen *et al*. 2003).

Once these stages of interphase were discovered a major goal of cell cycle research became the investigation of factors triggering the transitions between G_0, G_1, S, G_2 and M. The progression from G_2 to M phase was found to be an M phase-promoting factor, and this M phase-promoting factor was further discovered to be a cyclin-dependent protein kinase (CDK) (Nasmyth 1996). The CDKs are activated at different points of the cell cycle by shifting expression of the cyclins to progress the cell through the stages of the cell cycle (Vermeulen *et al*. 2003). The various actions of the cyclins and CDKs are as follows (Figure 1.2): cyclin D acts with CDK4 and CDK6 to progress the cell through the G_1 phase, cyclin E acts in conjunction with CDK2 to transition from G_1 to S phase, cyclin A acts with CDK2 to progress the cell through the S phase and with CDK1 to transition from G_2 to M phase, and cyclin B is involved with progression through the M phase of the cell cycle (Schwartz and Shah 2005; Vermeulen *et al*. 2003).

CDK activity is also controlled by a series of CDK inhibitors which can prevent progression through the cell cycle. There are two important families of CDK inhibitors: the INK4 family and the Cip/Kip family. The INK4 family of CDK inhibitors consists of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$. These INK4 proteins inhibit only G_1 phase CDKs and cyclins, namely: CDK4, CDK6 and cyclin D as well as the complexes formed through interaction of these CDKs and cyclins (Johnson and Walker 1999; Vermeulen *et al*. 2003). The Cip/Kip family proteins consist of p21, p27 and p57. These proteins broadly inhibit activity of CDKs and cyclin/CDK complexes, particularly involving

cyclin D or cyclin B, but show inhibitory activity on complexes involving the other cyclins as well (Johnson and Walker 1999; Vermeulen *et al*. 2003). The Cip protein p21 is also directly involved in DNA synthesis and is under the control of p53, the tumor suppressor protein (Johnson and Walker 1999; Vermeulen *et al*. 2003).

The final group of CDK inhibitory proteins important to this study are the RB proteins, pRBp107 and pRBp130. The RB proteins can bind to cyclin A/CDK2 and cyclin E/CDK2 complexes and inhibit their activity (Johnson and Walker 1999). The pRBp107 and pRBp130 proteins also bind to, and inhibit, proteins in the E2F family; these E2F proteins are involved in progression through the cell cycle by activating cyclins A and E (Johnson and Walker 1999; Mayol and Graña 1997).

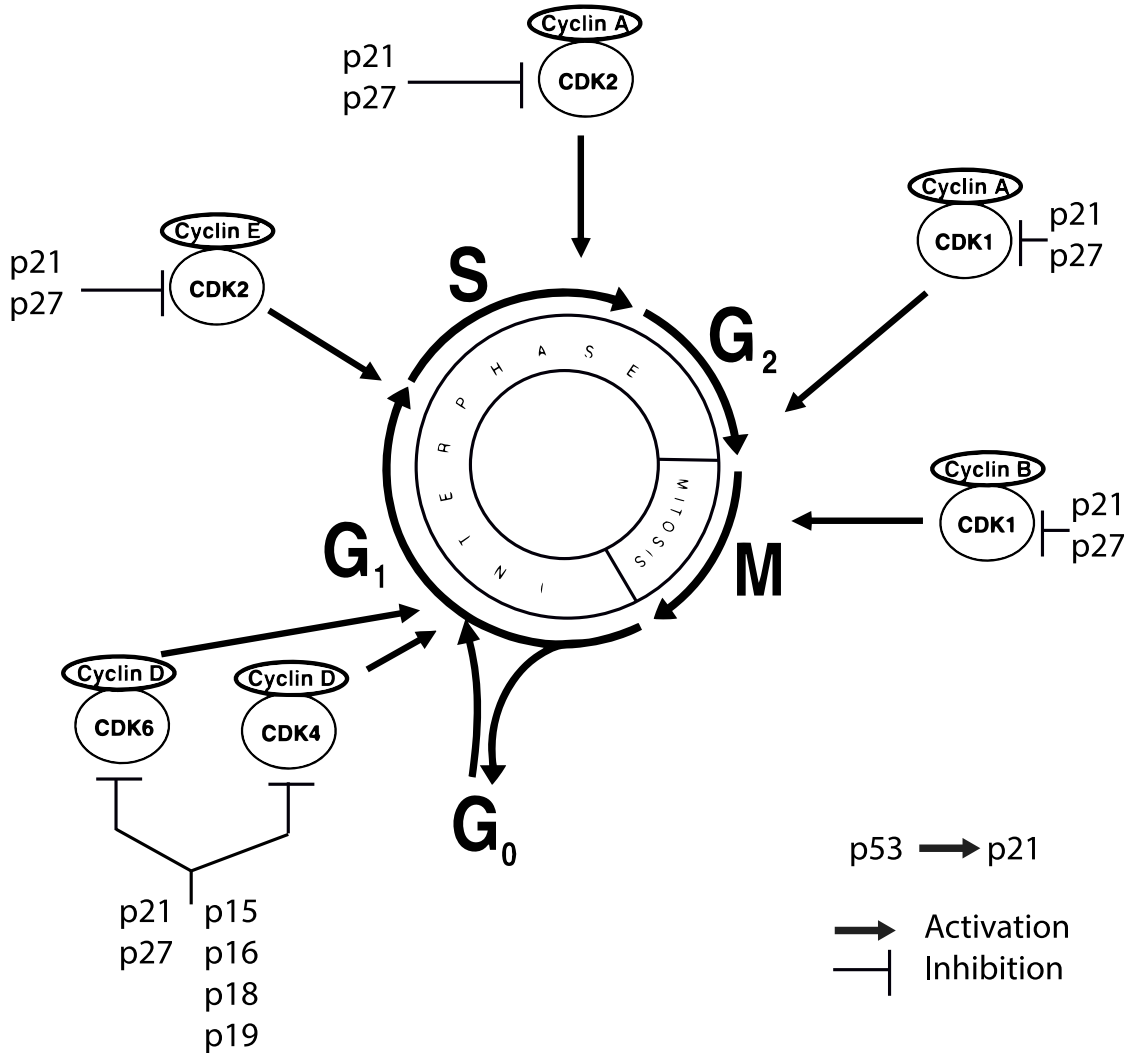

Figure 1.2: An overview of the cell cycle and the key regulatory proteins involved in cell cycle progression and arrest (Adapted from Vermeulen *et al*. 2003).

1.2.3.1 Dysregulation of the Cell Cycle in Cancer

Mutations to cell cycle proteins are common in many different kinds of cancer and roughly 70% of human prostate cancers exhibit mutations involving proteins of the cell cycle (Malumbres and Barbacid 2001). The most common mutations of cell cycle proteins in human prostate cancer involve the RB proteins, cyclin D1, cyclin E1, $p16^{INK4a}$ and p27 (Malumbres and Barbacid 2001). Of these affected proteins the most commonly mutated cell cycle protein across many different cancers is cyclin D1 (Johnson and Walker 1999; Vermeulen *et al*. 2003). Alterations in cyclin D1 activity can lead to quiescent cells progressing through G_1 and into S phase of the cell cycle. While the CDKs are rarely affected directly, alterations in CDK activity are common in many cancers as well due to CDK inhibitors being common sites of mutations in the development of cancer (Johnson and Walker 1999; Malumbres and Barbacid 2007, 2009; Vermeulen *et al*. 2003). This means that both CDKs themselves and CDK inhibitors are interesting targets for new anti-cancer drugs. One of the most well studied inhibitors of the cell cycle is the tumor suppressor protein p53. p53 acts through p21, meaning that a decrease in p53 activity would result in decreased p21 activity and therefore allow cell cycle progression to occur unmediated (Johnson and Walker 1999; Vermeulen *et al*. 2003). Of the three human prostate cancer cell lines used in this study, only DU145 cells show significant expression of p53, at roughly 90% that of normal prostate cells, while LNCaP cells express roughly 10% that of normal cells and PC3 cells do not express p53 at all (van Bokhoven *et al*. 2003).

1.2.4 Polyamine Biosynthesis and Catabolism

Polyamines were first discovered in 1678 by Anthonii van Leeuwenhoek, but no chemical formula or structure was known until 1924 (Dudley *et al*. 1924; van Leeuwenhoek 1678; Wallace *et al*. 2003). The polyamines are putrescine ($NH_2(CH_2)_4NH_2$), spermidine ($NH_2(CH_2)_3NH(CH_2)_4NH_2$) and spermine ($NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$) with spermidine and spermine being the most abundant in eukaryotic cells (Igarashi and Kashiwagi 2009). Due to the gap between discovery and characterization the names of spermine and spermidine refer to the original site of discovery of these two compounds while the name of putrescine refers to the abundance of the compound found in putrefying flesh (Wallace *et al*. 2003). Historically, polyamines were thought of as intracellular growth factors, but polyamines have more recently been found to regulate apoptosis as well (Wallace *et al*. 2003).

Polyamines are found in nearly all living species and are essential for cellular growth and proliferation, cell differentiation and the avoidance of apoptosis (Wallace *et al*. 2003). The major function of polyamines lies in their ability to bind to and change the conformation of DNA, RNA, ATP and proteins (Thomas and Thomas 2003). Polyamines bind to RNA and in doing so cause conformational changes in the RNA leading to increased protein synthesis (Igarashi and Kashiwagi 2009). Similarly, polyamines can bind to and stimulate the assembly of the 30S subunit of ribosomes to further contribute to increased protein synthesis (Igarashi and Kashiwagi 2009). Although polyamines interact more weakly with DNA and ATP, they can also bind to these molecules. By binding to DNA, polyamines can again cause conformational changes resulting in

increased transcription (Thomas and Thomas 2003). Polyamine binding to ATP allows ATP to more easily phosphorylate protein kinases and thereby drives enzymatic reactions (Igarashi and Kashiwagi 2009).

Polyamines have also been implicated in cellular signaling cascades, where decreased polyamine levels cause an increase in MAPK signaling which results in increased p21 expression and increased ornithine decarboxylase (ODC) expression (Thomas and Thomas 2003). This increase in ODC expression would then result in increased polyamine levels. Polyamines and ODC have also been shown to fluctuate during the cell cycle (Wallace *et al*. 2003). ODC expression and polyamine concentrations peak in G_1, prior to entering the S phase, and both peak again in G_2 prior to mitosis. The exact link between the polyamines and the cyclins/CDKs is unknown, but it is suspected that polyamines regulate cyclin degradation (Wallace *et al*. 2003). Polyamine content is tightly regulated by cells, maintaining the minimal concentration required for cell growth without inducing toxicity. Typically, polyamines are synthesized within the cell as needed, but polyamines can also be transported into and out of the cell to accommodate excess or deficiency (Wallace *et al*. 2003).

Polyamine biosynthesis (anabolism) and catabolism are controlled by a series of proteins summarized in Figure 1.3. Ornithine decarboxylase (ODC) is the rate limiting factor of polyamine biosynthesis, responsible for the conversion of ornithine to putrescine (Pegg 2006). ODC is itself regulated by antizyme (AZ) which is in turn regulated by antizyme inhibitor (AZI). AZ acts as a non-competitive inhibitor of ODC, where AZ and ODC will form a dimer which neutralizes ODC activity and increases the rate of ODC

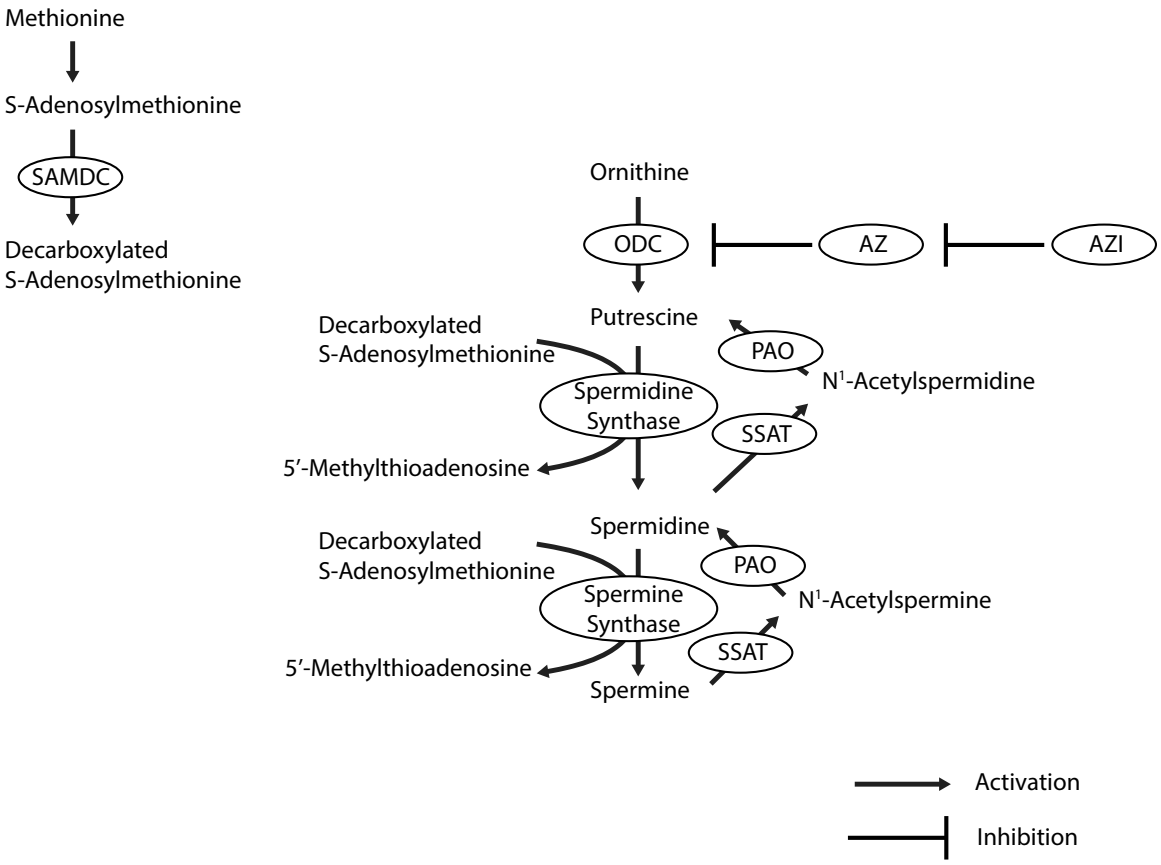

Figure 1.3: An overview of polyamine biosynthesis and catabolism and the key regulatory proteins and compounds involved (Copyright Scott McKeown 2014, used with permission).

degradation (Kahana 2009; Pegg 2006). AZI is structurally similar to ODC but lacks any ODC activity while being equally as effective at binding to AZ; AZI therefore functionally removes AZ from the system without decreasing ODC activity (Olsen and Zetter 2011; Pegg 2006). AZ is also capable of stimulating the transport of polyamines out of the cell and inhibits the transport of polyamines into the cell (Igarashi and Kashiwagi 2009).

While ODC activity is the essential primary step in polyamine biosynthesis there are other key enzymes involved. Continuing on the polyamine biosynthetic pathway, S-adenosylmethionine decarboxylase (SAMDC) is the rate limiting factor governing biosynthesis of spermidine from putrescine and of spermine from spermidine. SAMDC converts S-adenosylmethionine to decarboxylated S-adenosylmethionine which is then used by spermidine synthase and spermine synthase to synthesize spermidine and spermine, respectively (Shantz and Pegg 1999). In this way SAMDC controls spermidine and spermine production.

In polyamine catabolism there are two key proteins involved, polyamine oxidase (PAO) and spermidine/spermine N^1-acetyltransferase (SSAT), which act together to catabolize spermidine to spermine and spermine to putrescine. SSAT first acts to convert spermine to N^1-acetylspermine then PAO completes the catabolic process by converting N^1-acetylspermine to spermidine, this process is then repeated with SSAT converting spermidine to N^1-acetylspermidine and PAO converting this to putrescine (Casero and Pegg 1993; Shantz and Pegg 1999). SSAT is generally held to be the rate limiting factor in polyamine catabolism (Casero and Pegg 1993).

1.2.4.1 Dysregulation of Polyamine Biosynthesis and Catabolism

Polyamines are an obvious target for anti-cancer research due to their effects on cellular proliferation and the increased concentrations of polyamines in most types of cancer. Increased levels of polyamines have long been associated with many cancers, including prostate cancer, and often polyamine levels increase due to dysregulation of oncogenes and tumor suppressor genes (Nowotarski *et al*. 2013; Paz *et al*. 2014). It has been posited that polyamines themselves are a class of oncometabolites which are necessary for oncogenesis and that polyamine targeted therapy is a viable method of both chemoprevention and chemotherapy (Paz *et al*. 2014). Attempts at targeting the polyamine biosynthetic pathway have been made as early as 1972 when a SAMDC inhibitor was synthesized; these early attempts, however, proved unsuccessful as the compounds developed were toxic to many patients (Thomas and Thomas 2003; Williams-Ashman and Schenone 1972). More recently ODC has been described as an oncogene due to its role in cellular proliferation and ODC activity has become the target of therapies seeking to lower polyamine concentrations in cancer cells (Olsen and Zetter 2011).

Increased ODC expression occurs in many different types of cancer, with ODC being regulated by androgens in the normal prostate gland leading to highly elevated ODC levels in androgen-independent prostate cancer (Olsen and Zetter 2011; Pegg 2006). It has been found that, *in vitro*, ODC overexpression alone can lead to carcinogenesis and tumor formation and several ODC inhibitors are currently undergoing clinical trials for cancer treatment (Olsen and Zetter 2011). These clinical trials have not

been met with much success thus far, but the targeting of AZI in cancer therapy may also prove a sound strategy (Olsen and Zetter 2011). Increased AZI is associated with decreased patient survival and increased metastatic potential in several different types of cancer (Kahana 2009; Olsen and Zetter 2011). The disruption of AZI has been associated with inhibited ODC and decreased polyamine levels leading to a reduction in cellular growth and proliferation (Kahana 2009).

1.2.5 Cellular Signaling Pathways

The cellular signaling pathways mediate cellular response to both external and internal stimuli. For the purpose of this thesis the phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and activator protein-1 (AP-1) pathways will be focused upon.

1.2.5.1 PI3K/Akt Signaling Pathway

The phosphatidylinositol 3-kinase (PI3K) pathway (Figure 1.4) first became a focus in cancer research when it was associated with the activity of viral oncogenes in 1985 (Vivanco and Sawyers 2002; Whitman *et al*. 1985). In normal cells the PI3K pathway is an important regulator of cellular proliferation, growth, apoptosis and cytoskeletal rearrangement (Vivanco and Sawyers 2002). PI3K is a heterodimer consisting of PI3Kp85, the regulatory subunit, and PI3Kp110, the catalytic subunit; these PI3Kp85/ PI3Kp110 heterodimers typically exist pre-formed and inactive within the cell, awaiting signaling for activation (Vivanco and Sawyers 2003; Yuan and Cantley 2008). PI3K can be activated by either receptor tyrosine kinase (RTK) embedded in the cell membrane or by the RAS oncogene; this activation results in the PI3K mediated phosphorylation of phosphatidylinositol-4,5-biphosphate (PIP_2) which converts it to phosphatidylinositol-3,4,5-triphosphate (PIP_3) (Vivanco and Sawyers 2002; Yuan and Cantley 2008). PI3K activity is counteracted by the phosphatase and tensin homologue (PTEN) protein which converts PIP_3 back to PIP_2 and thereby acts as both an anti-signaling and tumor-suppressor gene (Vivanco and Sawyers 2002). PIP_3 then activates protein kinase B (Akt) which is in turn responsible for further signaling to the proteins responsible for proliferation, growth and apoptosis. Briefly, Akt is capable of activating IκB Kinase (IKK) and the mammalian target of rapamycin (mTOR) protein, which inhibit apoptosis and induce cellular growth respectively, and deactivating p53, p21, p27 and glycogen synthase kinase-3β (GSK3β) which results in the induction of cellular

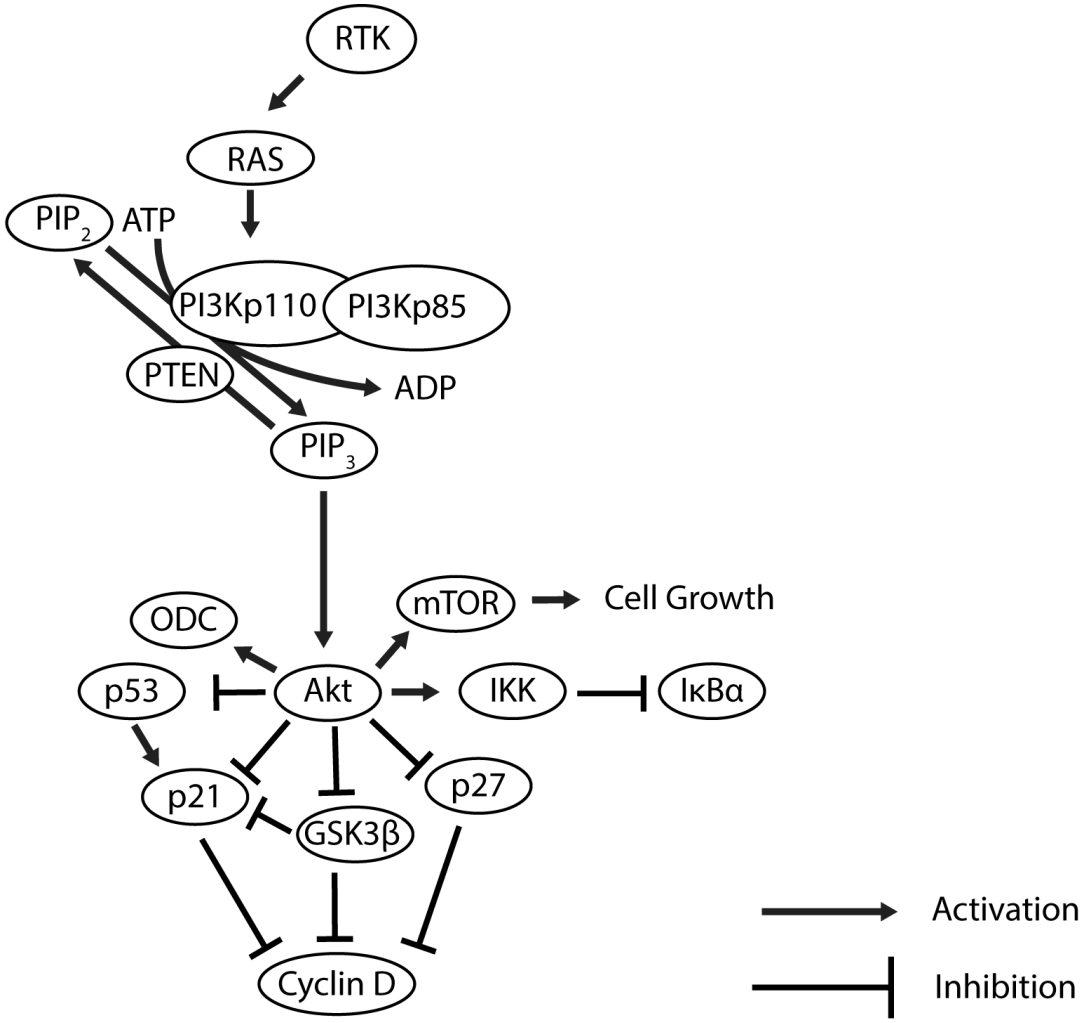

Figure 1.4: An overview of the PI3K/Akt signaling pathway and the key proteins involved (Copyright Scott McKeown 2014, used with permission).

proliferation (Fresno Vara *et al*. 2004; Vivanco and Sawyers 2002). In leukemia cells the PI3K pathway has also been shown to lead to ODC activation (Flamigni *et al*. 1997).

In cancerous cells mutations of the PI3K pathway are common at every stage in the pathway and mutations of PI3K genes are found in a broad range of cancers (Fresno Vara *et al*. 2004; Vivanco and Sawyers 2002; Yuan and Cantley 2008). The most common alteration to PI3K proteins is typically amplification, particularly in the case of PI3Kp110 and Akt (Fresno Vara *et al*. 2004). The exception to this is PTEN which is commonly deleted or mutated in such a way as to become non-functional. Loss of PTEN activity is common in human cancers, particularly in human prostate cancer (Liu *et al*. 2014). Of the three cell lines commonly used in prostate cancer research DU145 cells show normal, unmodified PTEN while LNCaP cells exhibit a mutated and less functional PTEN and PC3 cells are PTEN deficient (Vlietstra *et al*. 1998). Recent studies have shown that compounds which target PI3K to reduce its activity can induce cell cycle arrest (Liu *et al*. 2014).

1.2.5.2 MAPK Signaling Pathways

The mitogen-activated protein kinase (MAPK) pathways (Figure 1.5) consist of three major branches: the extracellular signal-regulated kinase (ERK) pathway, the c-Jun N-terminal kinase (JNK) pathway and the p38 pathway. These three MAPK pathways are involved in signaling for cellular growth, differentiation and death, and are widespread cellular signaling mechanisms common in the regulation of all eukaryotic cells (Krishna and Narang 2008). There are several features shared by the MAPKs, leading to them commonly being grouped together: the three pathways share a three tiered signaling mechanism whereby mitogen-activated protein kinase kinase kinases (MAP3Ks) are activated in response to extracellular stimuli, these MAP3Ks then activate mitogen-activated protein kinase kinases (MAP2Ks) which in turn activate the three MAPK pathways and their subsequent downstream targets (Krishna and Narang 2008).

The first MAPK described was ERK, as a protein involved in cell cycle control in yeast, which is expressed in all tissues of the human body and plays an important role in cellular proliferation and apoptosis (Boulton *et al*. 1990; Krishna and Narang 2008). ERK signaling begins with RTK and/or G protein-coupled receptors (GPCRs) responding to extracellular stimuli and activating G proteins such as RAS which in turn activate the MAP3K tier proteins like Raf kinases (Krishna and Narang 2008). These MAP3K proteins then activate the MAP2K tier proteins mitogen-activated protein kinase/ extracellular signal-regulated kinase kinase (MEK)-1 and MEK-2 which in turn activate ERK-1 and ERK-2, two isoforms of ERK (Krishna and Narang 2008). Downstream targets of ERK include c-jun, c-fos and various proteins involved in cellular proliferation

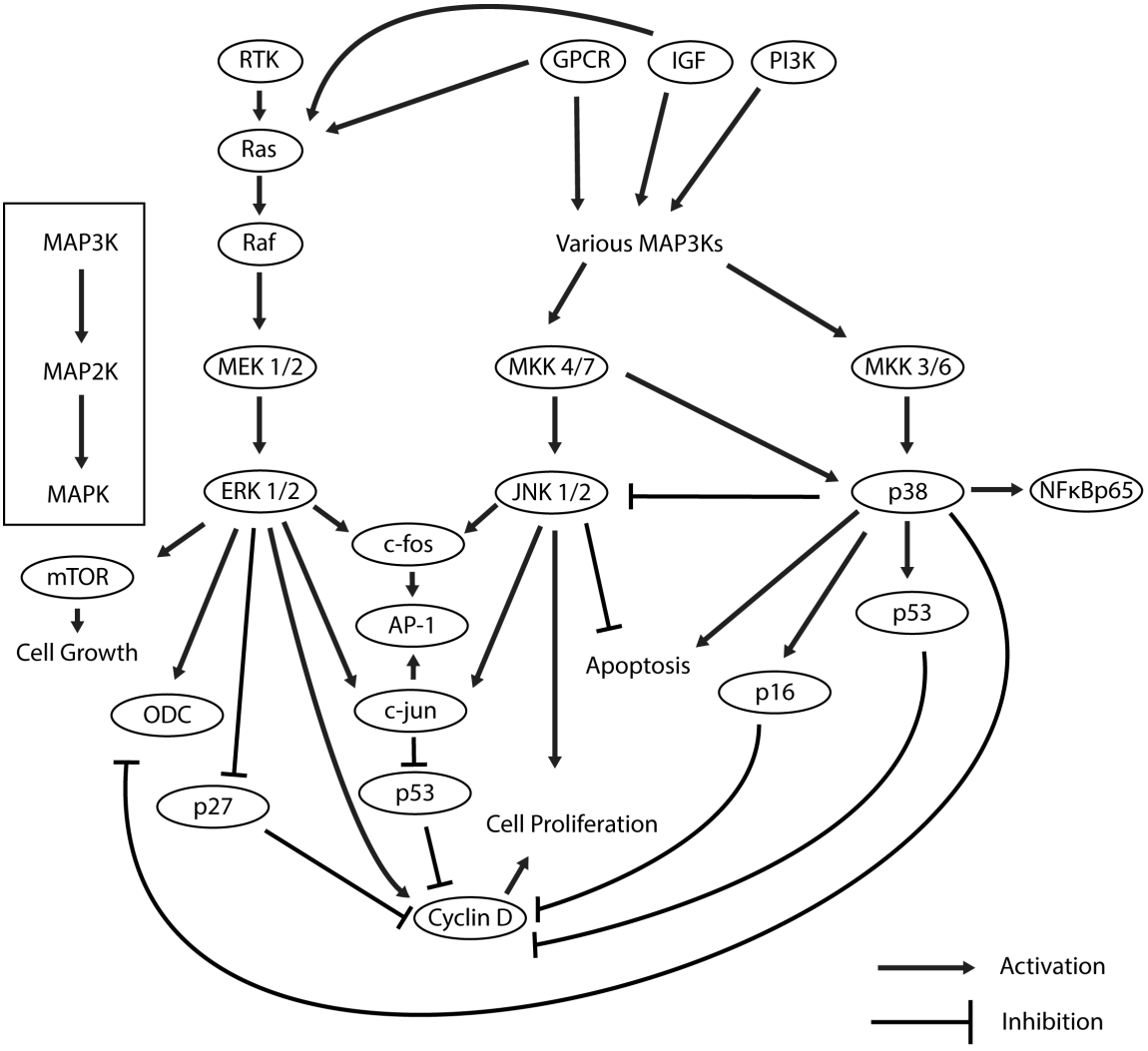

Figure 1.5: An overview of the MAPK signaling pathway and the key proteins involved (Copyright Scott McKeown 2014, used with permission).

and apoptosis (Krishna and Narang 2008). ERK dysregulation occurs in approximately 1/3 of all human cancers, where amplified ERK signaling leads to increased cellular proliferation, angiogenesis and metastasis (Krishna and Narang 2008). Increased expression of ERK has also been linked to increased ODC expression in human tissue cultures (Flamigni *et al*. 1999, 2001).

JNK was the second MAPK to be identified, originally known as stress activated protein kinase (SAPK) when first purified in rats, its name was later changed to JNK to better reflect its activity (Krishna and Narang 2008; Kyriakis and Avruch 1990). Like ERK, JNK exists in two different isoforms: JNK-1 and JNK-2. The JNK pathway is initially activated by a wide variety of external stimuli which in turn activate various MAP3Ks. These MAP3Ks then activate mitogen-activated protein kinase kinase (MKK)-4 and MKK-7 which in turn activate JNK-1 and JNK-2 (Krishna and Narang 2008). The major target of JNK is the activation of AP-1 through c-jun activation, although JNK can also inhibit p53, inhibit apoptosis and inhibit cellular proliferation (Krishna and Narang 2008; Wagner and Nebreda 2009; Weston and Davis 2007). JNK expression is greatly increased in several types of cancer, including prostate cancer (Krishna and Narang 2008; Wagner and Nebreda 2009; Weston and Davis 2007). JNK is capable of destabilizing p53, leading to its degradation, and loss of c-jun associated with decreased JNK signaling has been correlated with reduced tumor size and improved prognosis (Krishna and Narang 2008).

The effects of the third MAPK, p38, appear to be antagonistic to JNK, with the two MAPKs being intricately linked and acting in opposition (Wagner and Nebreda

2009). p38 was first identified as a 38 kDa protein rapidly phosphorylated in response to lipopolysaccharide exposure in a mouse cell line (Han *et al*. 1993; Ono and Han 2000). p38 is involved in osmoregulation, extracellular stress response and the cell cycle (Krishna and Narang 2008). Activation of p38 occurs by the same routes as JNK activation, with the same stimuli activation both JNK and p38 (Krishna and Narang 2008; Weston and Davis 2007). Separate from JNK, IGF can begin the MAP3K cascade toward p38 activation and there are two MAP2Ks, MKK-3 and MKK-6, which activate p38 (Krishna and Narang 2008). p38 can activate p53 and inhibitors of the cell cycle as well as downregulate cyclins, activate apoptosis and activate the NFκB p65 protein (Krishna and Narang 2008; Ono and Han 2000; Wagner and Nebreda 2009). There is also evidence to suggest that p38 can inhibit JNK signaling and that increased p38 is linked to decreased ODC expression (Flamigni *et al*. 2001; Wagner and Nebreda 2009). These factors support p38 as a tumor suppressor, with overexpression of p38 resulting in slowed cellular proliferation, and it has been suggested that p38 is necessary for induction of apoptosis (Krishna and Narang 2008).

1.2.5.3 NFκB and AP-1 Signaling Pathways

The final two signaling pathways discussed in this thesis are nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (Figure 1.6) and activator protein-1 (AP-1) (Figure 1.7). These two pathways act as transcription factors and migrate from the cytoplasm to the nucleus where they bind directly to DNA and influence protein expression (Fujioka *et al*. 2004). NFκB and AP-1 have been linked in that they are generally activated simultaneously and act in a cooperative manner (Fujioka *et al*. 2004).

NFκB was originally described coincidently by two independent research groups, one describing the NFκB subfamily of proteins which they identified in human HeLa cells and the other describing the reticuloendotheliosis (Rel) subfamily of proteins identified in chicken embryo fibroblasts (Gilmore 2006; Gilmore and Temin 1986; Sen and Baltimore 1986). The NFκB proteins were named after the κB site of DNA to which they bind (Gilmore 2006). The NFκB proteins are divided into these two subfamilies which form heterodimers using their shared Rel homology domain (RHD) (Gilmore 2006). The proteins of the NFκB subfamily are p50/p105 (NFκB1) and p52/p100 (NFκB2). The proteins of the Rel subfamily are NFκBp65 (RelA), RelB and c-Rel; these are the subunits responsible for activating transcription, with NFκBp65 being an important biomarker for human prostate cancer (Gannon *et al*. 2013; Gilmore 2006; Karin *et al*. 2002). NFκB proteins signal for the transcription of cyclin D, cyclin E, CDK2, MMPs, c-fos and other proteins involved in angiogenesis and apoptosis avoidance (Bassères and Baldwin 2006; Fujioka *et al*. 2004; Karin *et al*. 2002). NFκB is

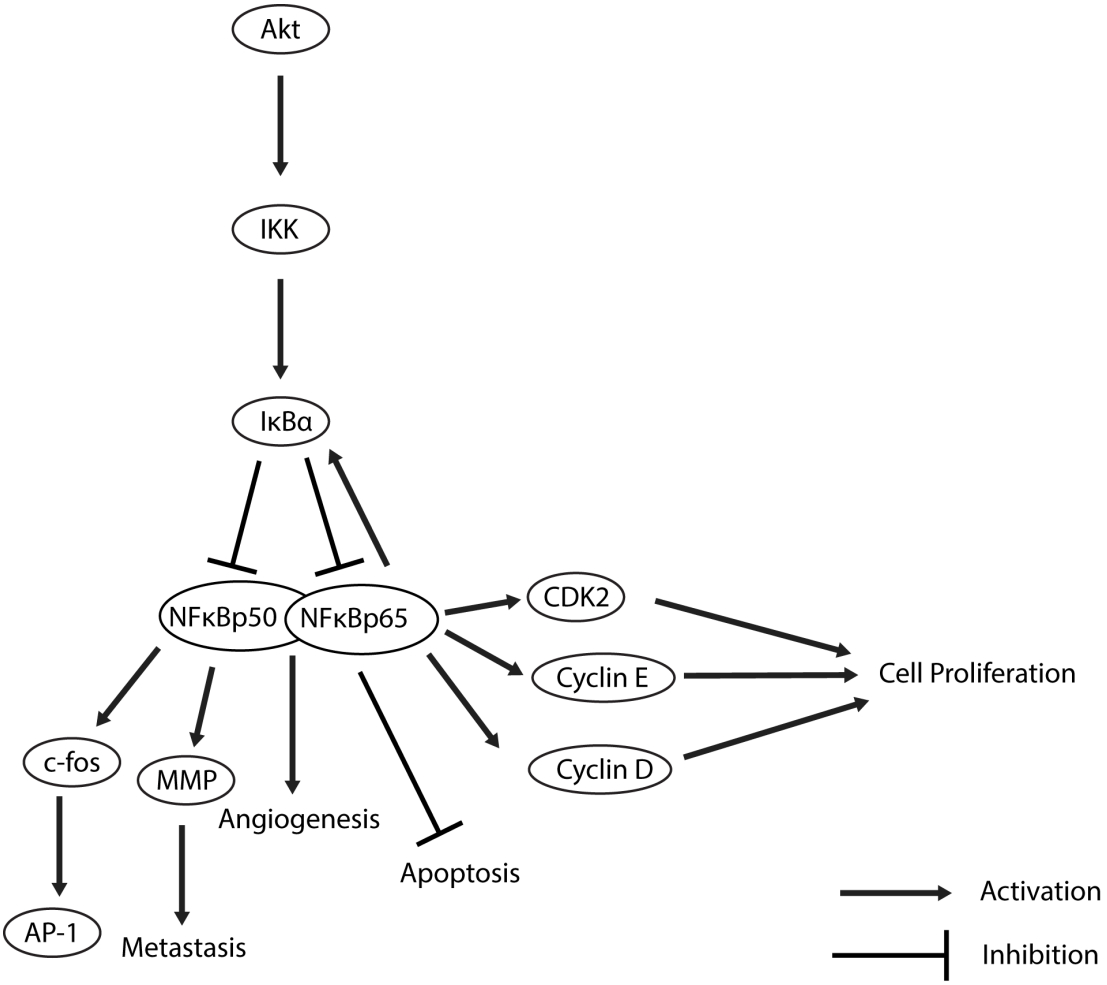

Figure 1.6: An overview of the NFκB signaling pathway and the key proteins involved (Copyright Scott McKeown 2014, used with permission).

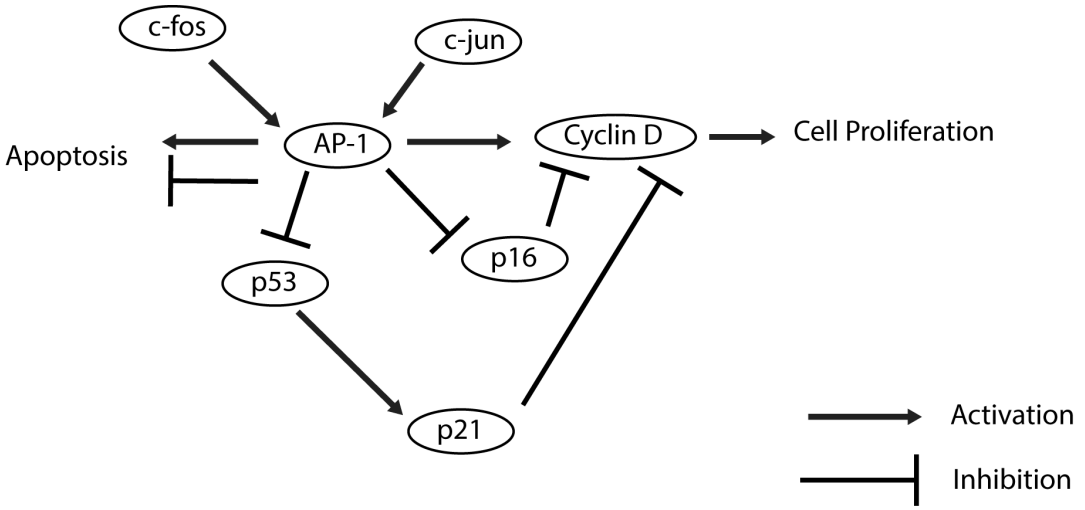

Figure 1.7: An overview of the AP-1 signaling pathway and the key proteins involved (Copyright Scott McKeown 2014, used with permission).

generally present in cells in its dimerized form, but is kept inactive by inhibitor of κB (IκB) proteins which recognize the RHD shared by NFκB proteins and bind to it, thereby blocking NFκB activity as the RHD is also used to bind DNA (Gilmore 2006; Karin *et al*. 2002). The most common of these IκBs is inhibitor of κB α (IκBα), which has the most universal ability to bind any NFκB protein (Gilmore 2006). IκB is in turn controlled by IκB Kinase (IKK) which degrades IκB and allows NFκB dimers to enter the nucleus and activate gene expression (Gilmore 2006). As NFκB dimers are common, in an inactive state due to IκB, it is IKK which generally initiates NFκB signaling. In normal cells, NFκB shows negative feedback by also increasing expression of IκBα (Gilmore 2006; Karin *et al*. 2002).

The AP-1 complex was first described by Lee *et al*. (1987) who were investigating eukaryotic transcription promotors. AP-1 proteins are divided among four subfamilies (jun, fos, mef and ATF) which form dimers to create the active AP-1 complex (Shaulian and Karin 2002). Of these four the jun and fos subfamilies are the most prevalent, with c-jun and c-fos being the most important proteins in each family (Shaulian and Karin 2001, 2002). The AP-1 transcription factor is generally activated by external stimuli and subsequent signaling through the MAPK pathway, with c-jun being the major target of JNK (Shaulian 2010; Shaulian and Karin 2002). AP-1 activates cycling D expression and inhibits the expression of p53, p21 and $p16^{INK4a}$ (Shaulian and Karin 2001, 2002). In cancer cells c-jun and c-fos overexpression is common, both being considered important oncoproteins, due to their role in transcription (Shaulian 2010; Shaulian and Karin 2001, 2002; Vesely *et al*. 2009). The role of AP-1 in apoptosis is more

complex, with AP-1 being capable of both induction and inhibition of apoptosis (Shaulian and Karin 2002). While the effect of AP-1 on apoptosis appears to be highly tissue specific, it is still not well understood.

1.2.6 Insulin-Like Growth Factors and Binding Proteins

Growth factors, such as insulin-like growth factor (IGF), cause cells to grow and divide much as their name would imply. The IGF proteins were independently discovered by two separate research groups, one studying mitogenic properties of proteins and the other studying metabolic functions. The first publication naming IGF was published in 1978, but research on the IGF proteins began several years prior (Rinderknecht and Humbel 1978). The first group to report on these proteins named them the somatomedins, describing their mitogenic function and ability to promote sulfate uptake in cartilage (Daughaday *et al*. 1972; Hall 1972; Van Wyk *et al*. 1974). Of these proteins described, the most promising were somatomedin A and somatomedin C which were both controlled by growth hormone (GH) (Hall 1972; Van Wyk *et al*. 1974). The other research group, investigating growth and metabolism promoting proteins, described the non-suppressible insulin-like activity (NSILA) proteins in human serum (Zapf *et al*. 1978). When a NSILA protein was discovered to have structural homology with insulin it was re-named insulin-like growth factor (Humbel 1990; Rinderknecht and Humbel 1978; Russell *et al*. 1998). Finally, both somatomedin A and C were found to be structurally identical to IGF-I (Engberg *et al*. 1984; Klapper *et al*. 1983).

There are two different IGF proteins, IGF-I and IGF-II, which primarily act at different stages in development (Yu and Rohan 2000). IGF-I is generally produced in the liver in healthy individuals, but can also be produced in most other tissues of the body (Pollak 2008). IGF-I is capable of regulating cellular proliferation and apoptosis in relation to the diet by stimulating protein synthesis and increasing metabolism (Harvey *et*

al. 2013; Pollak *et al*. 2004; You *et al*. 2002; Yu and Rohan 2000). Generally, IGF-I levels increase from birth and reach their peak expression at puberty, with a general decline in IGF-I thereafter (Yu and Rohan 2000). This rise and fall of IGF-I concentration is primarily controlled by GH (Yu and Rohan 2000). IGF-II is key to embryonic and fetal development, but is gradually replaced by IGF-I after birth; despite this, IGF-II is present in higher concentrations than IGF-I in adults with IGF-I being considered the more important governor of post-natal growth (Yu and Rohan 2000).

The IGFs signal (Figure 1.8) by binding to insulin-like growth factor receptors (IGF-R) in the cell membrane (Pollak 2008; Pollak *et al*. 2004; Yu and Rohan 2000). As with the IGFs there are two IGF-Rs, IGF-IR and IGF-IIR, which serve different functions in IGF signaling. IGF-IIR, which only IGF-II binds to, has no function in IGF signaling and simply serves to remove IGF-II from the system by degradation (Pollak 2008; Yu and Rohan 2000). IGF-IR is central to IGF signaling, binding both IGF-I and IGF-II, as it is expressed in all normal tissues and serves to begin IGF-mediated signaling within the cell (Pollak 2008; Yu and Rohan 2000). IGF-IR then activates the insulin-receptor substrate (IRS) which goes on to activate the PI3K and MAPK signaling pathways (Pollak *et al*. 2004). As mentioned previously, IGF-I expression can be tied to diet, with caloric restriction having been shown to cause a decrease in IGF-I expression (Harvey *et al*. 2013; Pollak *et al*. 2004). This decreased IGF-I has been linked, through PI3K signaling, to a resulting decrease in NF-κBp65 expression and therefore reduced NFκB signaling (Harvey *et al*. 2013).

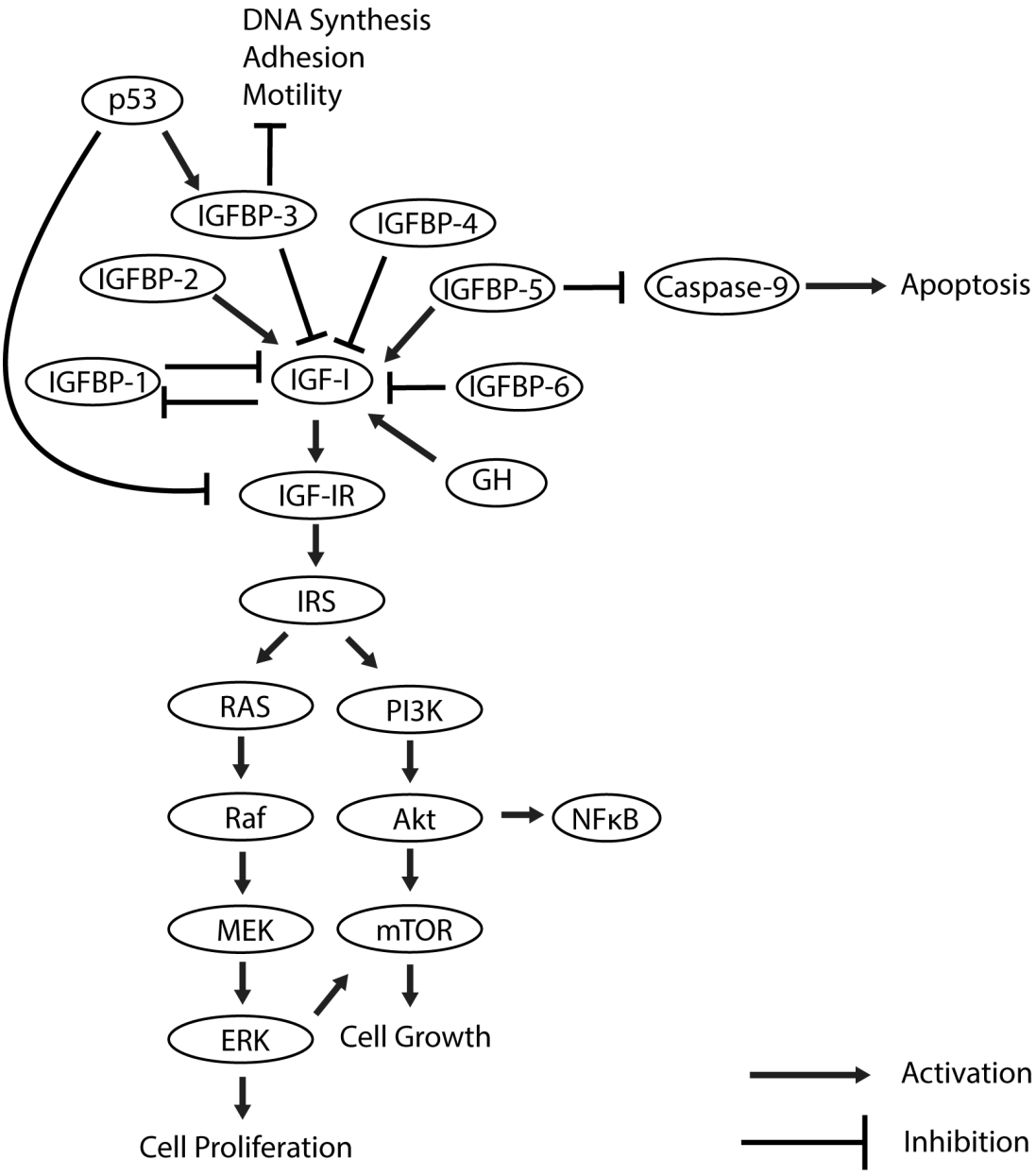

Figure 1.8 An overview of the IGF signaling pathway and the key proteins involved in its regulation (Copyright Scott McKeown 2014, used with permission).

Upstearm of IGF signaling are the six insulin-like growth factor binding proteins (IGFBPs). The IGFBPs were originally discovered while trying to purify IGF-I from serum, with the names of the IGFBPs reflecting the order in which they were discovered (Brown *et al*. 1989; Clemmons 1997; Drop *et al*. 1984; Hintz and Liu 1977; Martin and Baxter 1986; Póvoa *et al*. 1984; Shimasaki *et al*. 1991; Zapf *et al*. 1975). The IGFBPs function in binding to IGF before it can bind to IGF-IR. This serves to both stabilize IGF by slowing degradation as well as to functionally remove IGF from the system while the proteins remain bound together. IGF bioactivity can be decreased by increased expression of certain IGFBPs which are in turn activated by p53 (Pollak 2008). IGFBP-1 expression is typically limited to the liver, in low concentrations, and delivered to various tissues where it inhibits IGF-I by blocking interaction with IGF-IR (Clemmons 1997). IGFBP-2 through IGFBP-5 are commonly expressed in most cells at various concentration and are the most functionally important IGFBPs (Clemmons 1997). IGFBP-2 is the second most abundant IGFBP in serum, enhancing the effect of IGF-I (Clemmons 1997). There is also evidence of IGFBP-2 showing IGF-independent activity involved in the induction of mitosis (Firth and Baxter 2002). IGFBP-3 is the most common IGFBP and the principle carrier of IGF-I in serum, having one of the highest affinities for IGF-I (Clemmons 1997). Due to this high binding affinity IGFBP-3 inhibits IGF signaling by remaining bound to IGF-I; this tendency of IGFBP-3 to remain bound to IGF-I does, however, increase IGF-I half-life (Clemmons 1997; Massoner *et al*. 2009). Independent of IGF, IGFBP-3 can also directly inhibit DNA synthesis and cellular adhesion(Clemmons 1997). MMP-1 and MMP-2 have been known to degrade IGFBP-3 (Clemmons 1997; Massoner

et al. 2009). IGFBP-4 is the only IGFBP which exclusively inhibits IGF-I, resulting in decreased cell growth (Clemmons 1997; Durai *et al*. 2006). IGFBP-5, like IGFBP-2, stimulates IGF action and is commonly expressed in most cells (Clemmons 1997). The stimulation of IGF action happens due to a high affinity for IGFBP-5 to bind IGF-I in serum and a dramatic lowering of that binding affinity when IGFBP-5 binds to the extracellular matrix, resulting in the release of IGF-I nearby IGF-IR bound to the cell membrane (Clemmons 1997). IGFBP-5 can also induce mitosis and inhibit apoptosis independently of IGF (Cobb *et al*. 2004; Firth and Baxter 2002). Finally, IGFBP-6 is expressed in low concentrations, primarily by cells surrounding cartilage, and appears to primarily inhibit IGF-II, although some inhibition of IGF-I has been suggested (Clemmons 1997).

1.2.6.1 Dysregulation of Insulin-Like Growth Factors and Binding Proteins in Cancer

In cancerous cells IGF-I commonly becomes overexpressed, regardless of the tissue of neoplastic origin (Pollak 2008). For example, in a non-diseased state only stromal cells of the prostate will produce IGF, but cancerous prostate cells of any origin will overexpress IGF-I (Russell *et al*. 1998). IGF overexpression has been implicated with activation of many oncogenes, and drugs targeting IGF-I have been explored for their anti-cancer activity (Pollak 2008; Yu and Rohan 2000). High levels of IGF-I are frequently observed in prostate, breast and colorectal cancers and increased IGF-I is considered a risk factor for these diseases (Pollak 2008; Saikali *et al*. 2008; Yu and Rohan 2000). DU145, PC3 and LNCaP cells are all known to overexpress IGF-I (Russell *et al*. 1998). In DU145 cells addition of IGF-I to media was shown to increase cellular invasion through increased expression of MMP-2 and MMP-9, which was activated through the PI3K and MAPK pathways (Saikali *et al*. 2008). As with IGF-I, IGF-IR is also commonly overexpressed by human cancers, thus increasing signaling and activation of the PI3K and MAPK pathways; IGF-IIR, however, will act as a tumor suppressor by binding IGF-I without any associated signaling (Pollak 2008). Decreasing the expression of IGF-IR has also been shown to decrease cellular invasion in PC3 human prostate cancer cells (Saikali *et al*. 2008). Overexpression of IGF-IR is typical of aggressive tumors, with loss of PTEN having the capacity to cause excessive IGF-IR signaling (Pollak *et al*. 2004; Yu and Rohan 2000). IGF-IR expression can be suppressed by p53, suggesting that loss of p53 in cancerous cells contributes to increased IGF signaling (Yu and Rohan 2000).

The IGFBPs are also important in the development and regulation of cancerous cells. In prostate cancer cells increased expression of IGFBP-2 and IGFBP-5 is typical, as is decreased expression of IGFBP-3 (Yu and Rohan 2000). In PC3 and LNCaP cells, IGFBP-4 is also underexpressed (Russell *et al*. 1998). MMPs appear to be capable of degrading IGFBPs, resulting in the release of bound IGF-I (Pollak *et al*. 2004). Increased levels of IGFBP-2 are broadly found in prostate cancer, and increasing expression of IGFBP-2 has been associated with the progression toward androgen insensitivity (DeGraff *et al*. 2009). IGFBP-3 expression is typically decreased, and increased expression of IGFBP-3 has been shown to inhibit metastasis in mouse models of prostate cancer (Mehta *et al*. 2011). As p53 can induce IGFBP-3, this reduction of IGFBP-3 is associated with decreased p53 expression (Yu and Rohan 2000).

CHAPTER TWO

Magnolol Causes Alterations in the Cell Cycle in Androgen Insensitive Human Prostate Cancer Cells *In Vitro* by Affecting Expression of Key Cell Cycle Regulatory Proteins[1]

2.1 Introduction

Cancer is the second leading cause of death worldwide, with prostate cancer being the second most commonly diagnosed form in men (ACS 2011). A suggested approach in treating prostate cancer is to monitor the disease but not administer treatment until after symptoms have appeared due to the noncurative nature of treatment and associated side effects (Miyake *et al*. 2000; Raghavan *et al*. 1997). As a result, there has been an increasing amount of research interest in natural products and their bioactive effects, many of which have been used in traditional herbal medicine (Bemis *et al*. 2006; Ikarashi *et al*. 2001; Zhang *et al*. 2005). Magnolol is a lignan found in the roots and bark of the magnolia tree *Magnolia officinalis* and has been shown to have anti-cancer effects in human cells *in vitro* and *in vivo* (Chuang *et al*. 2011; Hsu *et al*. 2007; Hwang and Park 2010; Kim *et al*. 2007; Lee *et al*. 2009; Lee *et al*. 2008a; Lin *et al*. 2001; Lin *et al*. 2002; Rasul *et al*. 2012). In plants, lignans typically function as deterrents to pests, providing defense against insects by interfering with hormonal signaling (Harmatha and Dinan 2003; Lee and Xiao 2003). Lignans have also gained attention for their potential anti-

[1] The results of this study have been previously published in the journal Nutrition and Cancer (McKeown *et al*. 2014). This chapter is an adaptation and reformatting of that publication.

cancer and anti-viral effects, in both the plants which produce them and animals which consume them (Lee and Xiao 2003). Those studies performed *in vivo* suggest that magnolol remains active when administered at a sufficient oral dosage. Preclinical studies on the bioavailability of magnolol have previously been performed in mouse and rat models. Two methods of delivery were used in these previous studies, intravenous injection and oral administration. Of these two delivery methods it was found that an injected dose of 2-10 mg/kg could attain a maximum blood plasma concentration of 10 μg/ml or 40 μM magnolol, whereas oral administration at 20 mg/kg could attain a maximum blood plasma concentration of 0.1 μg/ml or 0.4 μM magnolol (Ho and Hong 2012; Lin *et al*. 2011; Tsai *et al*. 1996). The elimination half-life for magnolol was determined to be 15 min, with total body clearance at 72-75 ml/min/kg (Ho and Hong 2012; Homma *et al*. 1993; Lin *et al*. 2011). Preclinical studies on the toxicity of magnolol have also previously been performed, showing that magnolol was nontoxic and that no drug-related side effects nor alterations in immune response were observed at any dose including the highest tested concentration of 2.5 g/kg in mice, 0.48 g/kg/day for 21 days in rats or 0.24 g/kg/day for 90 days in rats (Ho and Hong 2012; Li *et al*. 2007; Liu *et al*. 2007). A clinical study has also shown that after oral treatment with a magnolol-containing herbal medicine, magnolol was retained in the blood plasma for up to 1 h; furthermore the total recovery of magnolol in urine was 16.7% of that administered after 24 h (Ho and Hong 2012; Homma *et al*. 1993). In the present study, the *in vitro* effect of magnolol on the cell cycle and related activities in DU145 and PC3 human prostate cancer cells was examined. This is novel research as the effects of magnolol on the cell

cycle in human prostate cancer cells have not previously been investigated. This study also represents the first evidence that magnolol can affect the expression of inhibitors of the cell cycle leading to cell cycle effects at later time points.

2.2 Materials and Methods

All antibodies used in this study were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). These include mouse polyclonal anti-actin and anti-$p16^{INK4a}$, and rabbit polyclonal anti-cyclin A, anti-cyclin B1, anti-cyclin D1, anti-cyclin E, anti-CDK2, anti-CDK4, anti-p21, anti-p27, anti-pRBp107 and anti-pRBp130. Magnolol was purchased from Sigma-Aldrich Canada (Oakville, ON). All other chemicals and materials were purchased from Sigma-Aldrich Canada unless otherwise indicated.

2.2.1 Cell Culture and Treatment with Magnolol

Human DU145 and PC3 prostate adenocarcinoma cells (ATCC, Manasses, VA) were cultured on 100 mm plastic tissue-culture dishes (Falcon, Mississauga, ON) in α-MEM and RPMI (Gibco, Burlington, ON), respectively, supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Invitrogen Canada, Burlington, ON), and were incubated at 37 ^{0}C. in 5% CO_2. At 70% confluence, cells were exposed to either 40 or 80 μM concentrations of magnolol, dissolved in dimethyl sulfoxide (DMSO), for 24 h or 6 h [concentration of DMSO in both magnolol-treated and control cells was 0.2% (v/v)]. Control cells received only DMSO. After treatment, cells were removed using trypsin diluted in phosphate buffered saline (PBS) and re-suspended in α-MEM with 10% FBS

and centrifuged for 5 min at 500x G. The medium was then removed by aspiration. The remaining cell pellet was resuspended and washed in cold PBS. Cells were again centrifuged for 5 min at 500x G. After centrifugation, the PBS was removed by aspiration and the pellet was then stored at -80 ^{0}C until further analysis.

2.2.2 Immunoblot Analysis

Cell pellets were resuspended in 100 µl of 10 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM PMSF, and briefly sonicated. Cell lysates were then centrifuged at 9300x G for 20 min at 4 ^{0}C. The supernatant was removed from the pellet and evaluated for protein content. Equal amounts of protein from this extract were mixed in a 3:1 ratio with standard Laemmli buffer consisting of 50 mM Tris-HCl (pH 6.8), 10% SDS, 0.1% bromophenol blue, 10% glycerol and 100 mM β-mercaptoethanol and then the samples were boiled for 3 min. Electrophoresis through 10% SDS-PAGE gels was used to resolve proteins which were then transferred onto nitrocellulose membranes (Bio-Rad, Mississauga, ON) by electro-blotting. Membranes were then incubated in the presence of a 1% BSA (w/v) TBS-Tween (0.05% v/v) solution overnight at 4 ^{0}C. Primary antibodies diluted to 1:200 (v/v) were then applied to membranes and incubated for 1 h at room temperature. After incubation, the membranes were washed 3 times with TBS-Tween (0.05% v/v) for a total of 30 min and then incubated with alkaline phosphatase (AP)-conjugated secondary antibodies (1:1000) for 1.25 h at room temperature. After incubation the membranes were again washed 3 times with TBS-Tween (0.05%) for a total of 30 min and rinsed quickly with distilled water, then exposed to SigmaFast BCIP-

NBT tablets (Sigma-Aldrich, Oakville, ON) dissolved in distilled water to visualize protein expression levels. Western blots were then photographed using Infinity Capture Software (Lumenera Corp., Ottawa, ON) and densitometry was performed with ImageJ Software (National Institute of Health, Bethesda, MD).

2.2.3 Alamar Blue Cytotoxicity Assay

Cellular viability after exposure to magnolol at 40 and 80 μM for 6 and 24 h was determined using the Alamar Blue assay (Invitrogen Canada) as per manufacturer's instruction. The Alamar Blue assay measures cellular viability through the indicator resazurin. Resazurin, a blue compound which is nonfluorescent, is reduced to resorufin, a pink compound which is highly fluorescent, by the metabolic activity of viable cells (O'Brien *et al*. 2000). Alamar Blue is advantageous to use because it is a nontoxic alternative to the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) cytotoxicity assay (O'Brien *et al*. 2000).

Briefly, cells were subcultured into a 96-well plate (Falcon) at approximately 5000 cells/well. After 24 h incubation at 37 ^{0}C in 5% CO_2, the cells were treated with magnolol for either 6 or 24 h. The control for this experiment consisted of cells treated with DMSO only [concentration of DMSO in magnolol-treated and control cells was 0.2% (v/v)]. A negative control consisting of wells containing media only (no cells, no magnolol and no DMSO) was also evaluated. Alamar Blue was added to each well, to a final concentration of 10%, 3 h prior to fluorescence measurement which allowed for 3 h

of exposure to Alamar Blue. After exposure to Alamar Blue, fluorescence was read with an excitation wavelength of 528 nm and an emission wavelength of 590 nm.

2.2.4 Flow Cytometry

Flow cytometric analysis was performed on cells treated with 40 and 80 μM magnolol for 24 h and control cells treated with DMSO only [concentration of DMSO in both magnolol-treated cells and control cells was 0.2% (v/v)]. Flow cytometry can be used to measure cellular DNA content (Nunez 2001). To accomplish this, propidium iodide is used to stain DNA. The propidium iodide bound to DNA will fluoresce, and this fluorescence can be measured to determine DNA content (Nunez 2001). The flow cytometer will measure the cells within a sample and graph the recorded fluorescence from each of the cells individucally (Nunez 2001). This graph representing the DNA content of every individual cell within the sample can then be used to identify the proportion of cells in the various phases of the cell cycle (Nunez 2001). A peak representing cells which have unpaired DNA shows the percentage of cells in the G_0/G_1-phase of the cell cycle (Nunez 2001). A separate peak representing cells cells which have paired DNA shows the percentage of cells in the G_2/M-phase of the cell cycle (Nunez 2001). Finally, cells which do not fall into either of these categories, fluorescing somewhere between the peaks characteristic of unpaired and paired DNA, are representative of the percentage of cells in the S-phase of the cell cycle (Nunez 2001).

Cells were cultured and centrifuged as described above and placed in 225 μl of 70:30 ethanol:PBS and incubated on ice overnight. “Fixed” cells were then centrifuged

for 5 min at 200x G at 4 ^{0}C, and were resuspended in 250 µl of ice-cold PBS and then incubated for 5 min. Cells were then centrifuged for 5 min at 200x G at 4 ^{0}C and the cell pellet was suspended in a PBS-propidium iodide [at a concentration of 0.02 mg/ml (w/v))]staining solution containing Triton-X [at a concentration of 0.088% (v/v)] and 0.4 mg/ml of RNAse and incubated for 30 min at room temperature in the dark. After incubation the cells were transferred to a round bottom 96-well plate (Falcon), and fluorescence measured using a BD FACSARRAY bioanalyzer equipped with BD FACSARRAY system software version 1.0.3 (BD Biosciences, Mississauga, ON). Analysis was performed using WinMDI software version 2.9 (Scripps Reseach Institute, La Jolla, CA).

2.2.5 Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 4.03 for Windows (GraphPad Software, Inc. San Diago, CA). Results of immunoblot analyses and cytotoxicity assay were compared using a 1-way analysis of variance (ANOVA) with a Tukey's post hoc test and results were considered statistically significant at $P \leq 0.05$.

2.3 Results

2.3.1 Magnolol Induced Cytotoxicity

The effect of magnolol on DU145 and PC3 human prostate cancer cells was measured using the Alamar Blue cytotoxicity assay. Viability of DU145 cells decreased significantly at both 40 and 80 μM after 6 and 24 h (Figure 2.1). In DU145 cells, viability decreased by 30% and 60% at 40 and 80 μM respectively after 6 h of exposure to magnolol and by 49% and 76% at 40 and 80 μM respectively after 24 h exposure to magnolol. Viability of PC3 cells also decreased at 80 μM after 6 and 24 h, decreasing to 50% and 48%, respectively (Figure 2.1). No statistically significant change in PC3 viability was observed at 40 μM magnolol at either time point.

2.3.2 Magnolol Affects Cell Cycle Progression

Flow cytometric analysis was performed to determine if magnolol affects cell cycle progression by measuring the proportion of cells at each stage of the cell cycle. As shown in Figures 2.2, 2.3 and 2.4, DU145 and PC3 cells exposed to 40 and 80 μM magnolol for 24 h show alterations in the cell cycle, with an increased number of cells at G_0/G_1-phase and a decreased number of cells at S and G_2/M-phases. In control DU145 cells 42%, 36% and 22% of cells were in the G_0/G_1, S and G_2/M-phase of the cell cycle, respectively. DU145 cells exposed to 40 μM magnolol exhibited 50%, 29% and 21% distribution between the G_0/G_1, S and G_2/M-phase of the cell cycle, respectively. Finally, DU145 cells exposed to 80 μM magnolol exhibited 50%, 33% and 17% distribution between the

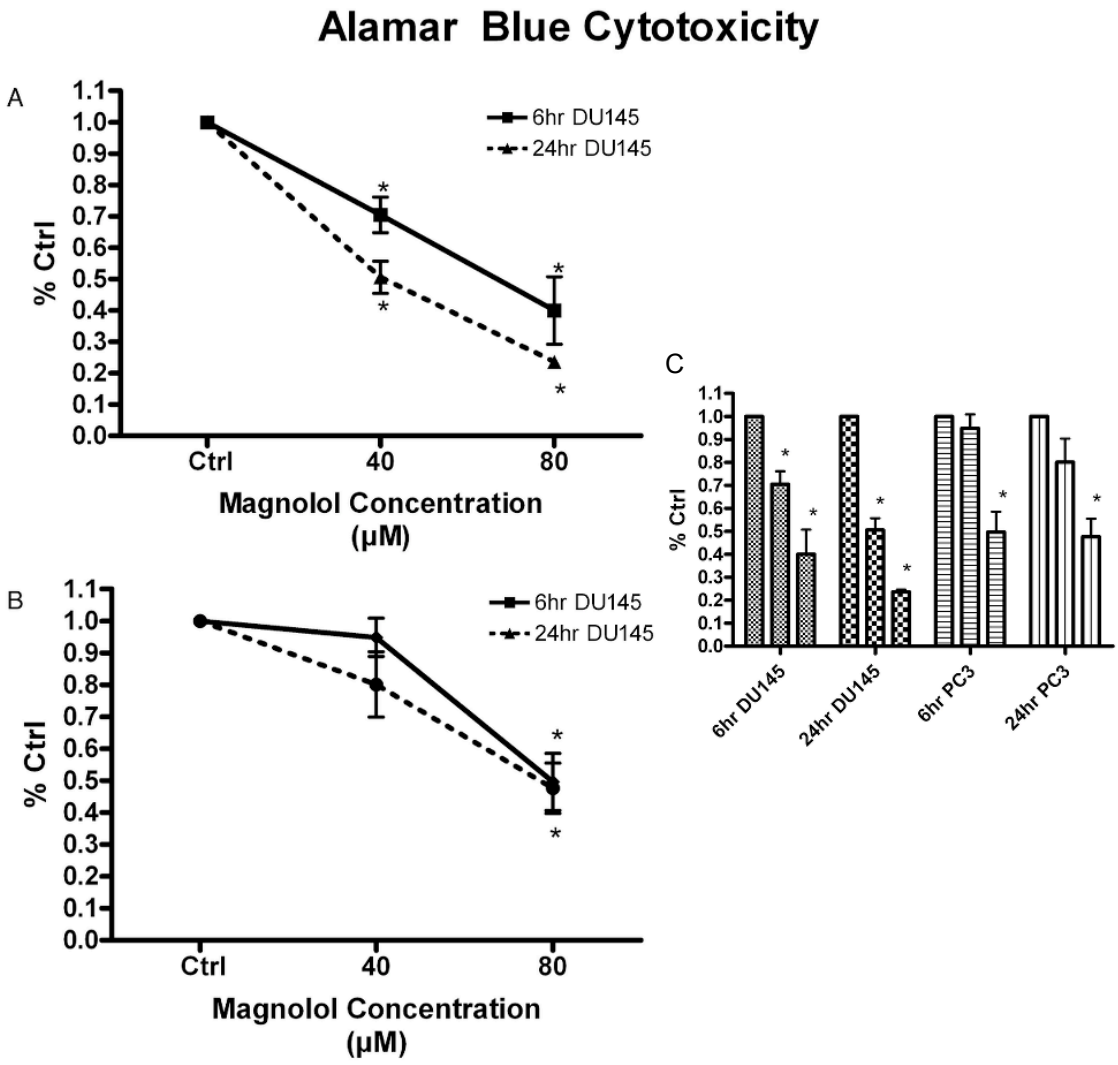

Figure 2.1: Magnolol is cytotoxic to DU145 (A) and PC3 (B) cells *in vitro*. Cells were treated with either DMSO (Ctrl) or 40 or 80 μM magnolol in DMSO for either 6 or 24 h and cytotoxicity was determined by Alamar Blue assay. Fluorescence was read at 528 nm (excitation) and 590 nm (emission) wavelengths. Control cells were set to 100% viability. (C) Bar graph representing and comparing the data presented in figures A and B, where the first column of each group represents control, the middle column represents 40 μM and the final column represents 80 μM magnolol in DMSO. All experiments were performed with 6 replications using separate samples. (*) indicates $P \leq 0.05$.

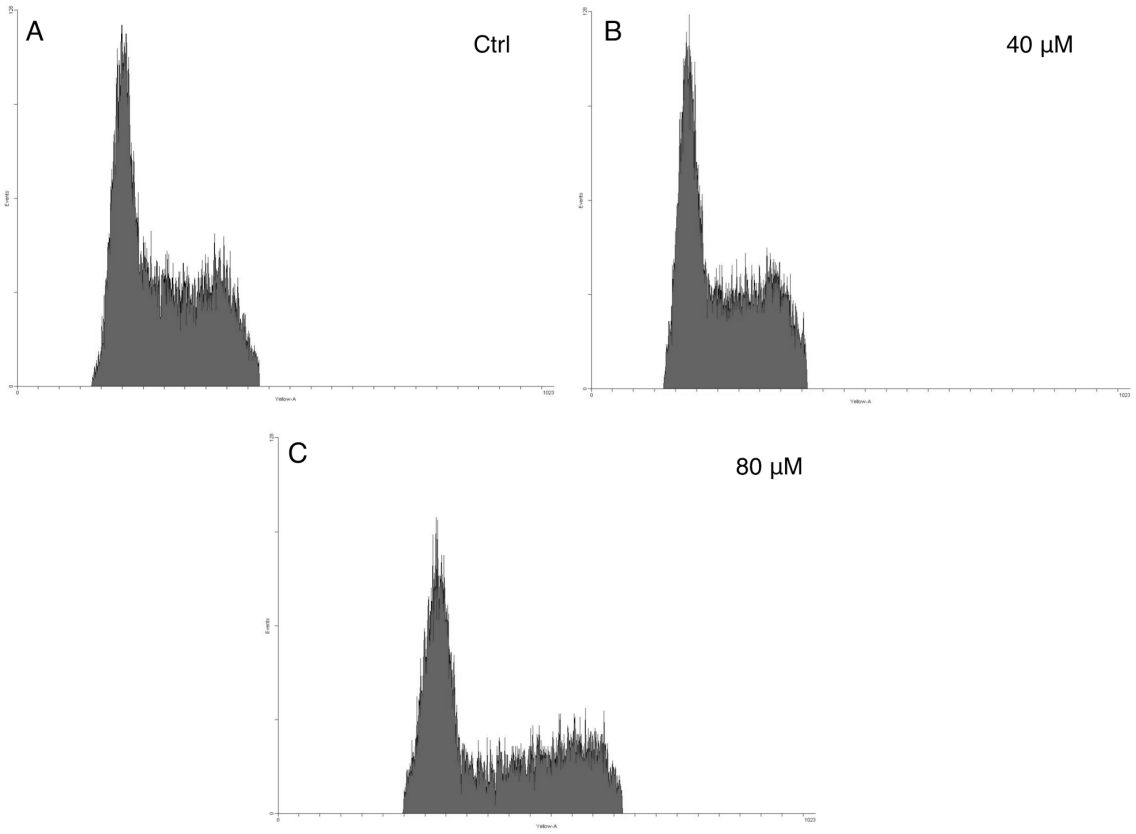

Figure 2.2: Magnolol causes alterations in the cell cycle in DU145 cells *in vitro*. Figure 2.2A, B and C are representative of flow cytometric analysis performed on DU145 cells treated with either DMSO (Ctrl), 40 or 80 µM magnolol in DMSO, respectively.

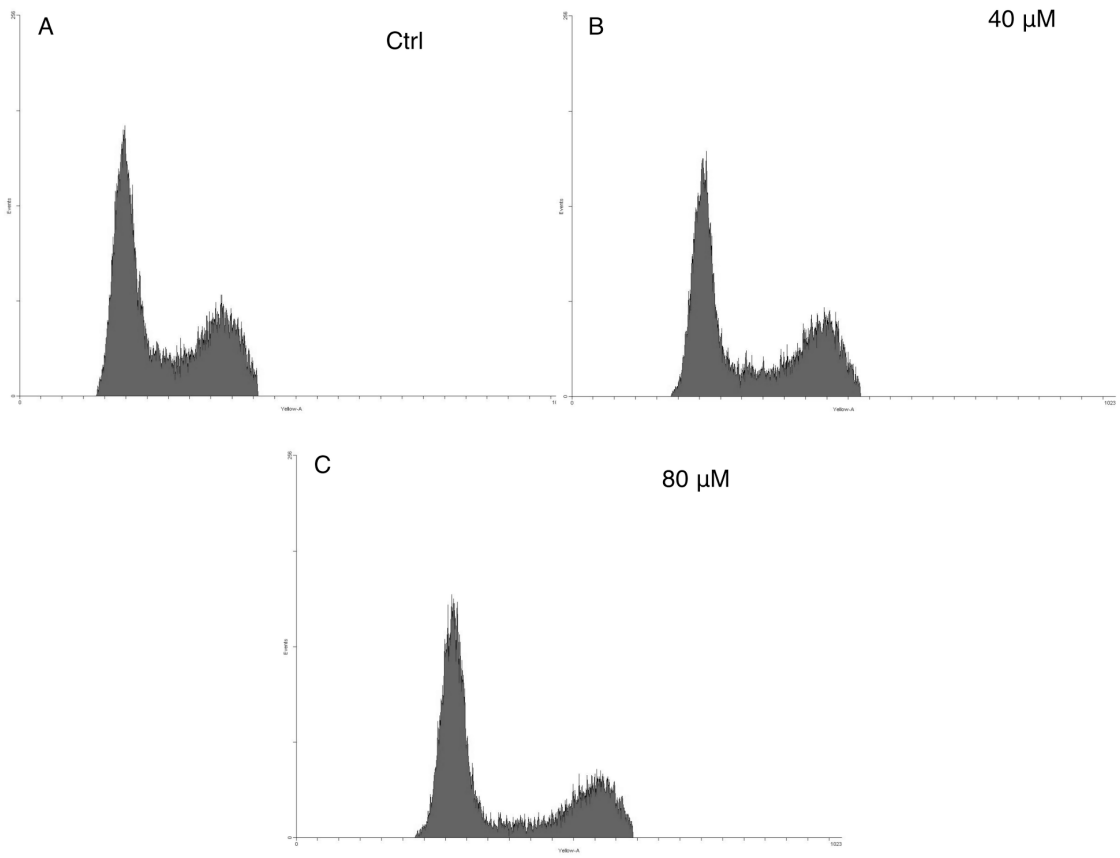

Figure 2.3: Magnolol causes alterations in the cell cycle in PC3 cells *in vitro*. Figure 2.3A, B and C are representative of flow cytometric analysis performed on PC3 cells treated with either DMSO (Ctrl), 40 or 80 µM magnolol in DMSO, respectively.

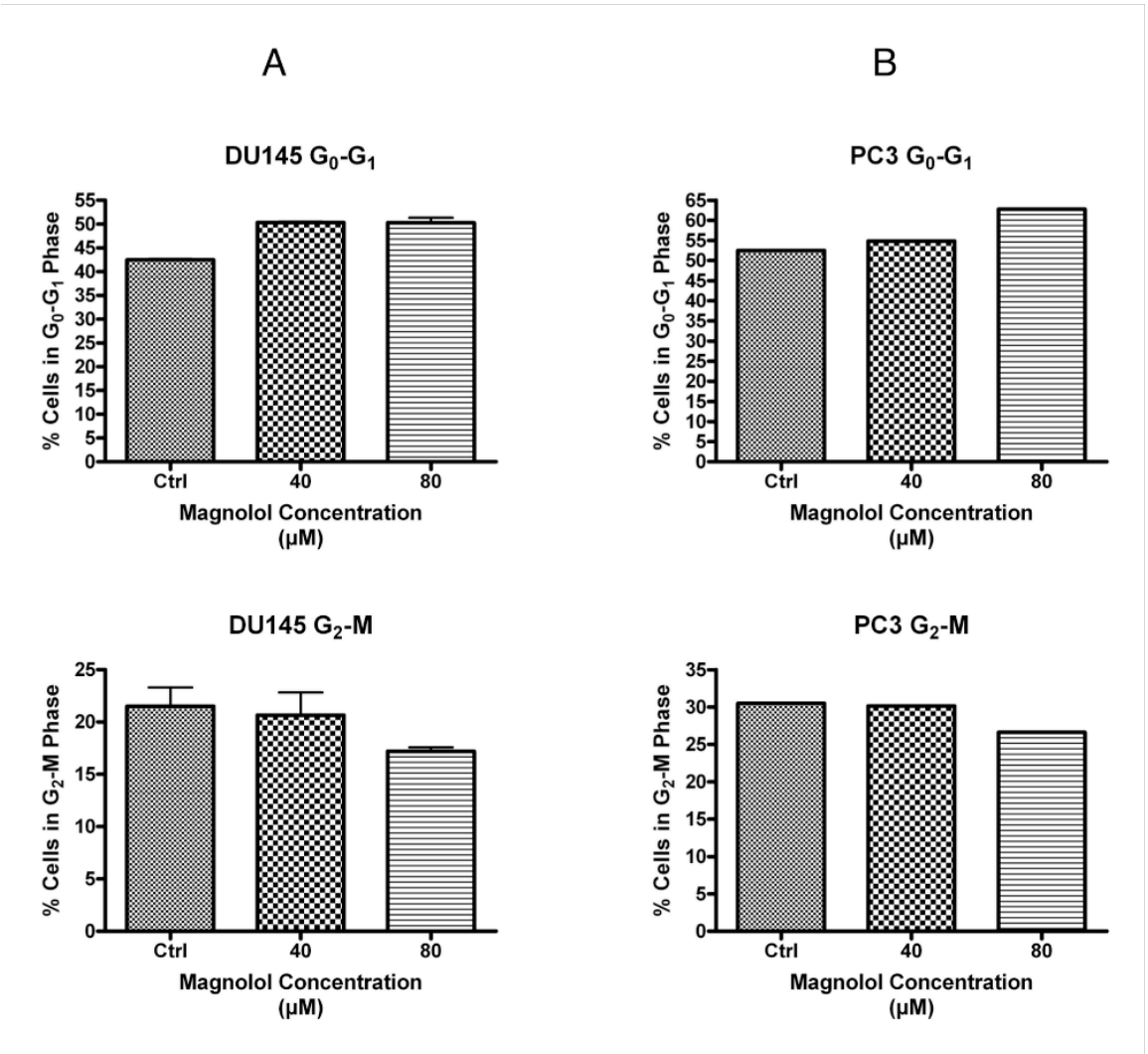

Figure 2.4: Bar Graphs indicating the results of cell cycle analysis performed on DU145 (A) and PC3 (B) cells at concentrations of magnolol indicated. These histograms summarize information presented in Figures 2.2 and 2.3. DU145 data (A) represents the average of two experiments, while PC3 data (B) represents the results of a single experiment. As such, no statistical analysis was performed.

G_0/G_1, S and G_2/M-phase of the cell cycle, respectively. In control PC3 cells 53%, 17% and 30% of cells were in the G_0/G_1, S and G_2/M-phase of the cell cycle, respectively. PC3 cells exposed to 40 μM magnolol exhibited 55%, 15% and 30% distribution between the G_0/G_1, S and G_2/M-phase of the cell cycle, respectively. Finally, PC3 cells exposed to 80 μM magnolol exhibited 63%, 11% and 26% distribution between the G_0/G_1, S and G_2/M-phase of the cell cycle, respectively.

2.3.3 Magnolol Affects Cell Cycle Protein Expression

In DU145 cells (Figure 2.5) the expression of CDK2 and CDK4, as well as cyclins A, B1, D1 and E decreased in response to 80 μM magnolol after 24 h while in PC3 cells (Figure 2.6) the expression of CDK2 as well as cyclins A and D1 decreased in response to 80 μM magnolol after 24 h. In DU145 cells, CDK2 expression significantly decreased by approximately 62% after exposure to 80 μM magnolol for 24 h, but were unchanged at 40 μM for 24 h. CDK4 expression decreased by approximately 22% and 55% in DU145 cells exposed to 40 and 80 μM magnolol for 24 h, respectively, with a statistically significant change at 80 μM. In PC3 cells a statistically significant decrease in CDK2 expression, by 61%, was observed after 24 h exposure to 80 μM magnolol, but remained unchanged at 40 μM. No significant changes were observed in CDK4 expression. Cyclin A protein expression levels were decreased by 55% and 75%, cyclin B1 by 29% and 66%, cyclin D1 by 15% and 65% and cyclin E by 42% and 64% in DU145 cells exposed to 40 and 80 μM magnolol for 24 h, respectively, with all changes statistically significant

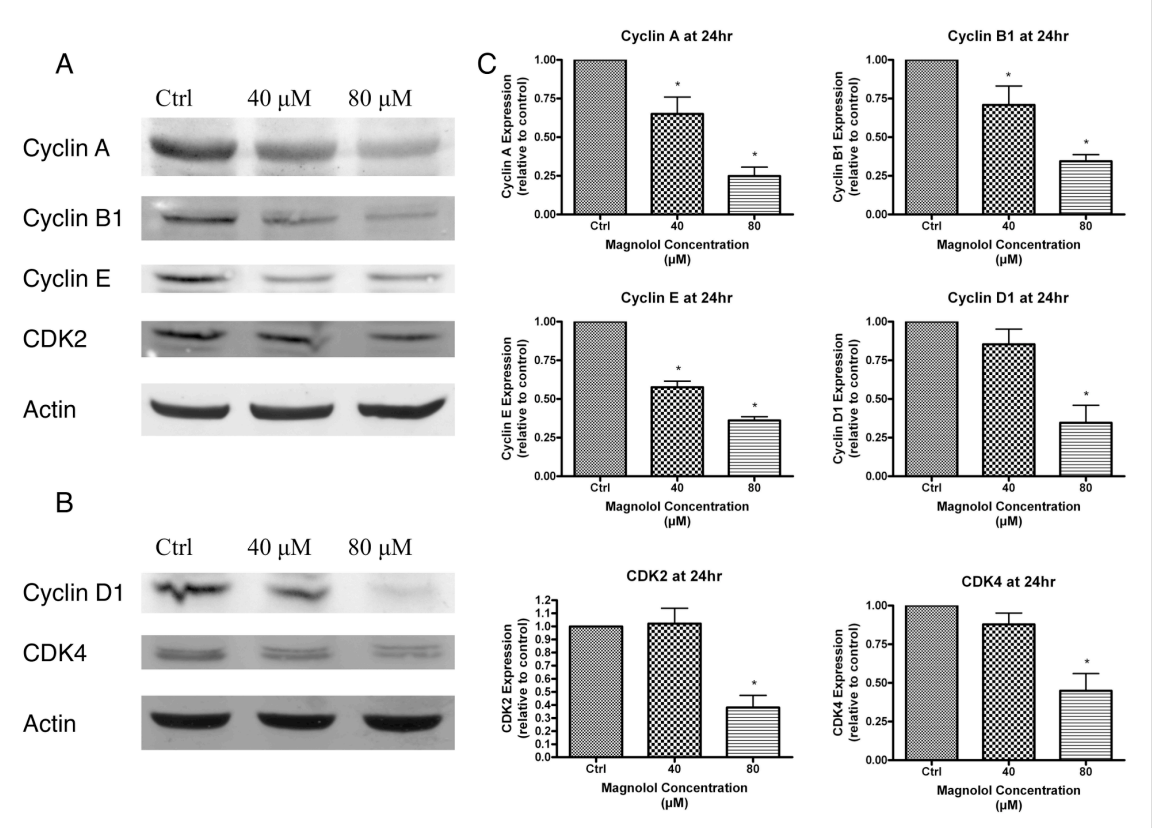

Figure 2.5: Magnolol affects expression of cell cycle proteins in DU145 cells *in vitro*. Cells were treated with either DMSO (Ctrl), 40 or 80 μM magnolol in DMSO as indicated for 24 h. Actin was used as a loading control (the actin used in Figure 2.5A is the same as that used in Figure 2.7A as these samples were run concurrently). Each blot shown is representative of results obtained from at least three replications using separate samples. (*) indicates statistical significance at $P \leq 0.05$.

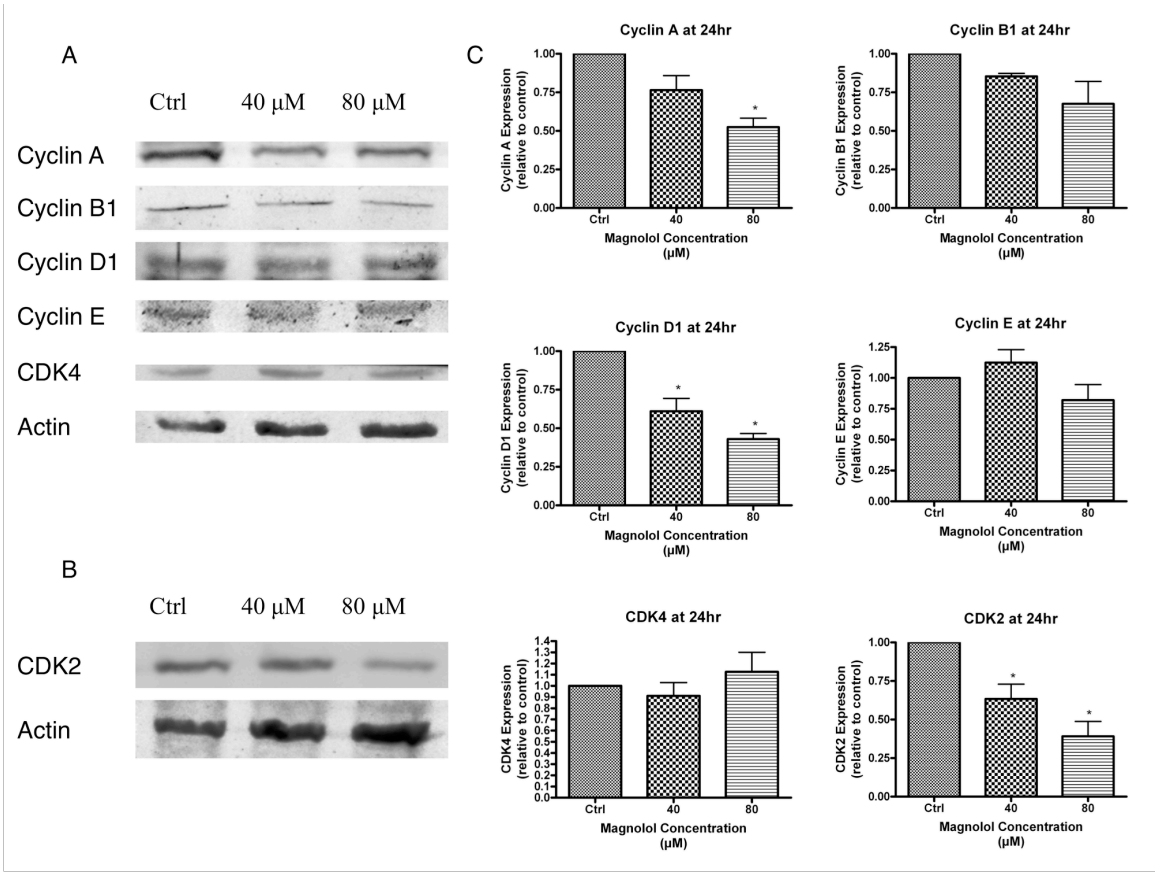

Figure 2.6: Magnolol affects expression of cell cycle proteins in PC3 cells *in vitro*. Cells were treated with either DMSO (Ctrl), 40 or 80 μM magnolol in DMSO as indicated for 24 h. Actin was used as a loading control (the actins used in Figure 2.6A and 2.6B are the same as those used in Figure 2.8A and 2.8B, respectively, as these samples were run concurrently). Each blot shown is representative of results obtained from at least three replications using separate samples. (*) indicates statistical significance at $P \leq 0.05$.

except for the change to cyclin D1 at 40 μM. In PC3 cells, the only statistically significant changes observed were a reduction of cyclin A expression by 48% after exposure to 80 μM magnolol for 24 h and a reduction in cyclin D1 expression by 39% and 57% after exposure to 40 and 80 μM magnolol, respectively, for 24 h.

To further determine why expression of these key cell cycle proteins were affected by magnolol exposure in DU145 (Figure 2.7) and PC3 cells (Figure 2.8), protein expression levels of cell cycle inhibitors, specifically $p16^{INK4a}$, p21, p27, pRBp107 and pRBp130, were also measured. No statistically significant change was observed in expression of $p16^{INK4a}$, p21 or p27 in DU145 cells exposed to 40 or 80 μM magnolol for 24 h. Statistically significant changes in protein expression were only observed in pRBp107 in PC3 cells. Protein expression of pRBp107 decreased by 47% and 88% in DU145 cells exposed to 40 and 80 μM magnolol, respectively, for 24 h. In PC3 cells, expression of pRBp107 decreased by 54% and 47% after 24 h exposure to 40 and 80 μM, respectively. Protein expression of pRBp130 increased by 29% in DU145 cells exposed to 40 μM magnolol, and increased by 15% in cells exposed to 80 μM magnolol for 24 h.

As alterations in the expression of p21 and p27 can occur relatively early and then enact changes in the cell cycle at later time points (Lee *et al*. 2008a), expression of these two proteins was examined after 6 h exposure to 40 and 80 μM magnolol in DU145 (Figure 2.9) and PC3 cells (Figure 2.10). Expression of p21 increased by 5% and 15% after exposure to 40 and 80 μM magnolol, respectively, in DU145 cells after 6 h. Expression on p21 also increased by 49% and 43% after exposure to 40 and 80 μM magnolol, respectively, in PC3 cells after 6 h. Protein expression of p27 remained

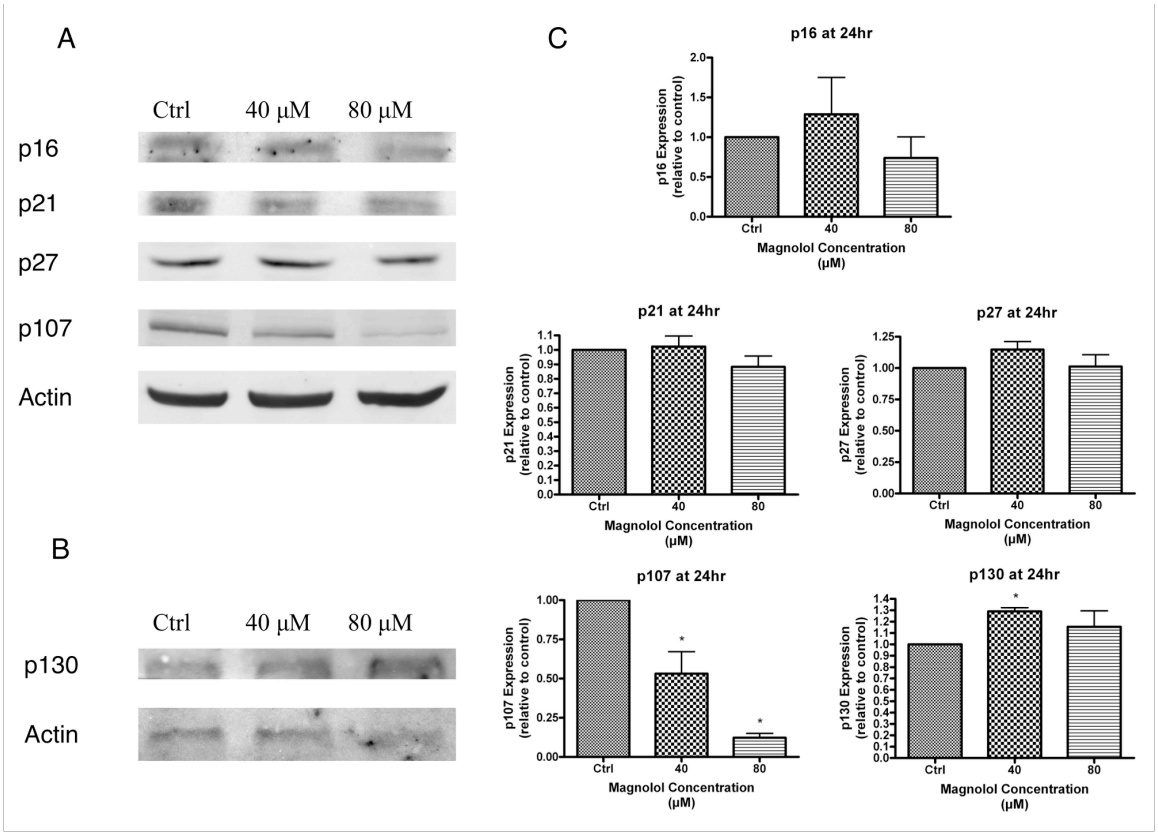

Figure 2.7: Magnolol affects expression of cell cycle inhibitors in DU145 cells *in vitro*. Cells were treated with either DMSO (Ctrl), 40 or 80 μM magnolol in DMSO as indicated for 24 h. Actin was used as a loading control (the actin used in Figure 2.7A is the same as that used in Figure 2.5A as these samples were run concurrently). Each blot shown is representative of results obtained from at least three replications using separate samples. (*) indicates statistical significance at $P \leq 0.05$.

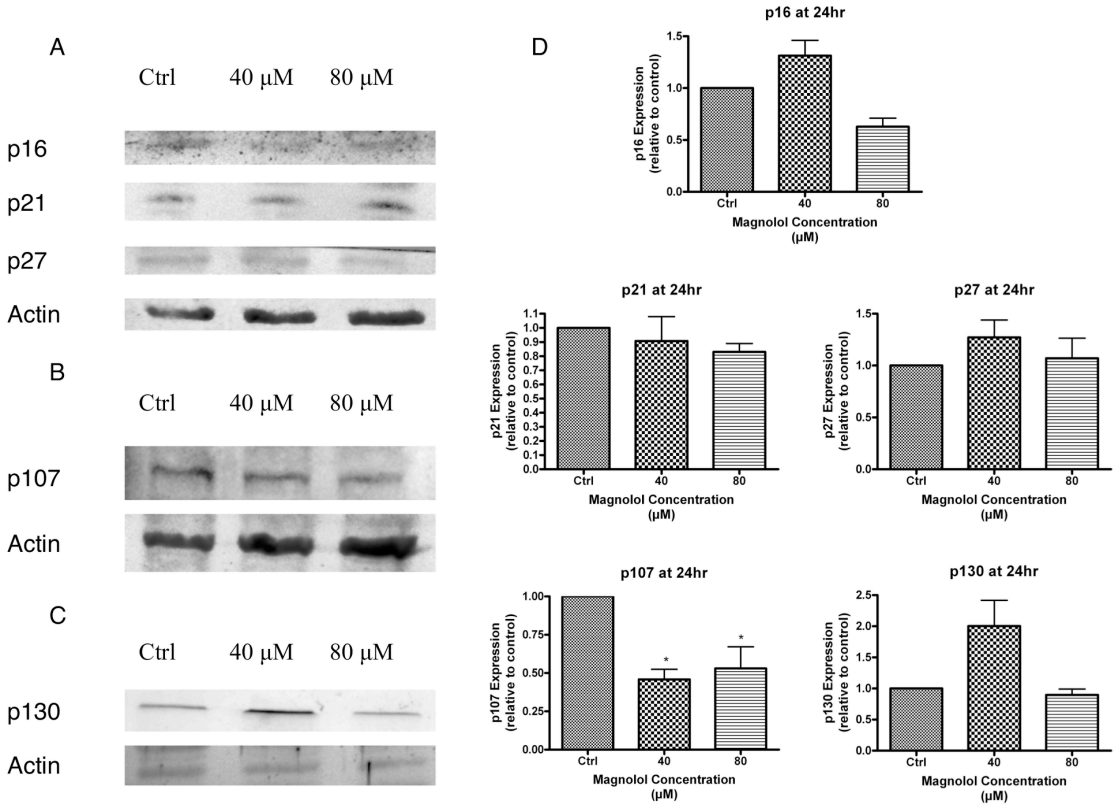

Figure 2.8: Magnolol affects expression of cell cycle inhibitors in PC3 cells *in vitro*. Cells were treated with either DMSO (Ctrl), 40 or 80 μM magnolol in DMSO as indicated for 24 h. Actin was used as a loading control (the actins used in Figure 2.8A and 2.8B are the same as those used in Figure 2.6A and 2.6B, respectively, as these samples were run concurrently). Each blot shown is representative of results obtained from at least three replications using separate samples. (*) indicates statistical significance at $P \leq 0.05$.

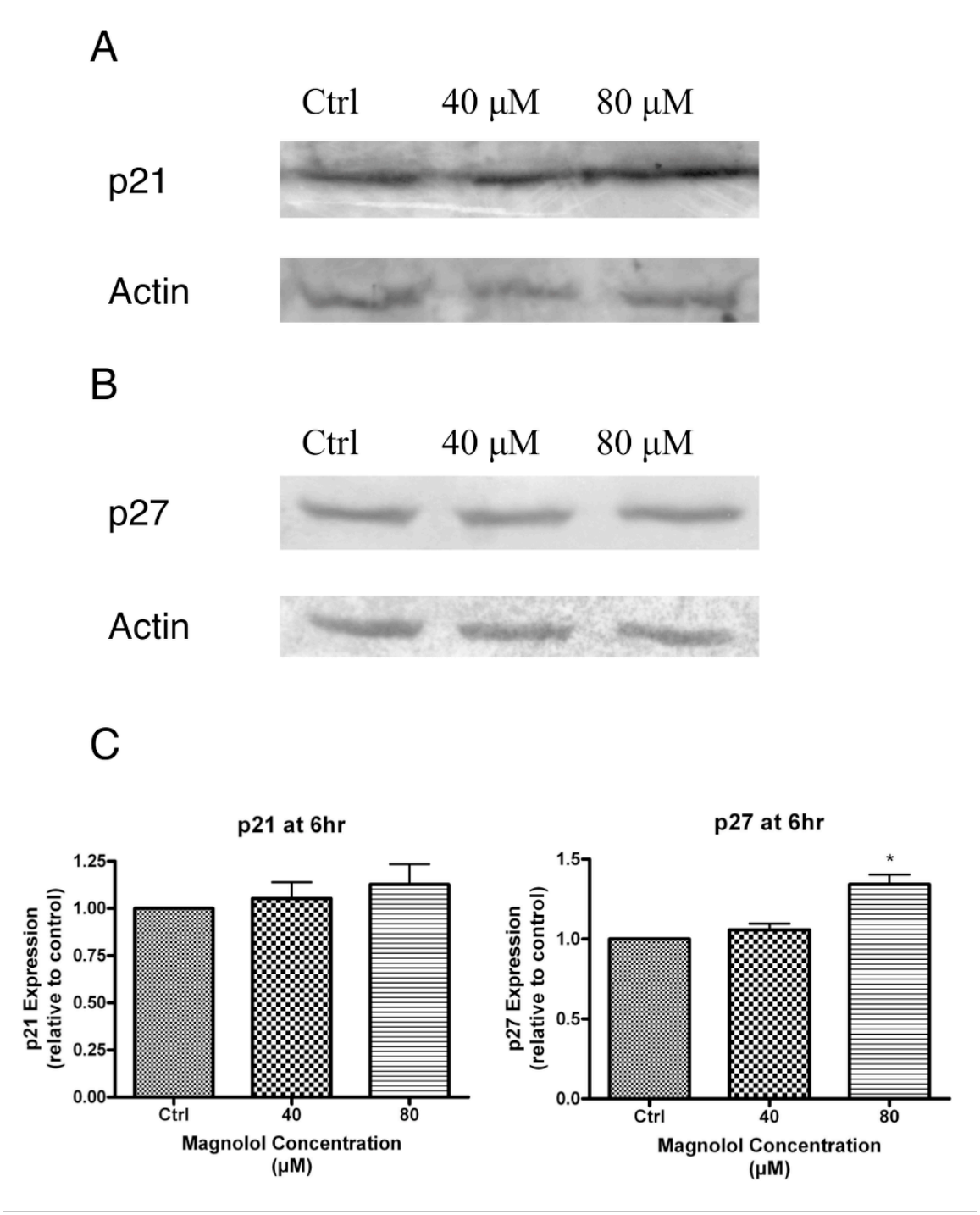

Figure 2.9: Magnolol affects expression of p21 and p27 after 6 h in DU145 cells *in vitro*. Cells were treated with either DMSO (Ctrl), 40 or 80 μM magnolol in DMSO as indicated for 6 h. Actin was used as a loading control. Each blot shown is representative of results obtained from at least three replications using separate samples. (*) indicates statistical significance at $P \leq 0.05$.

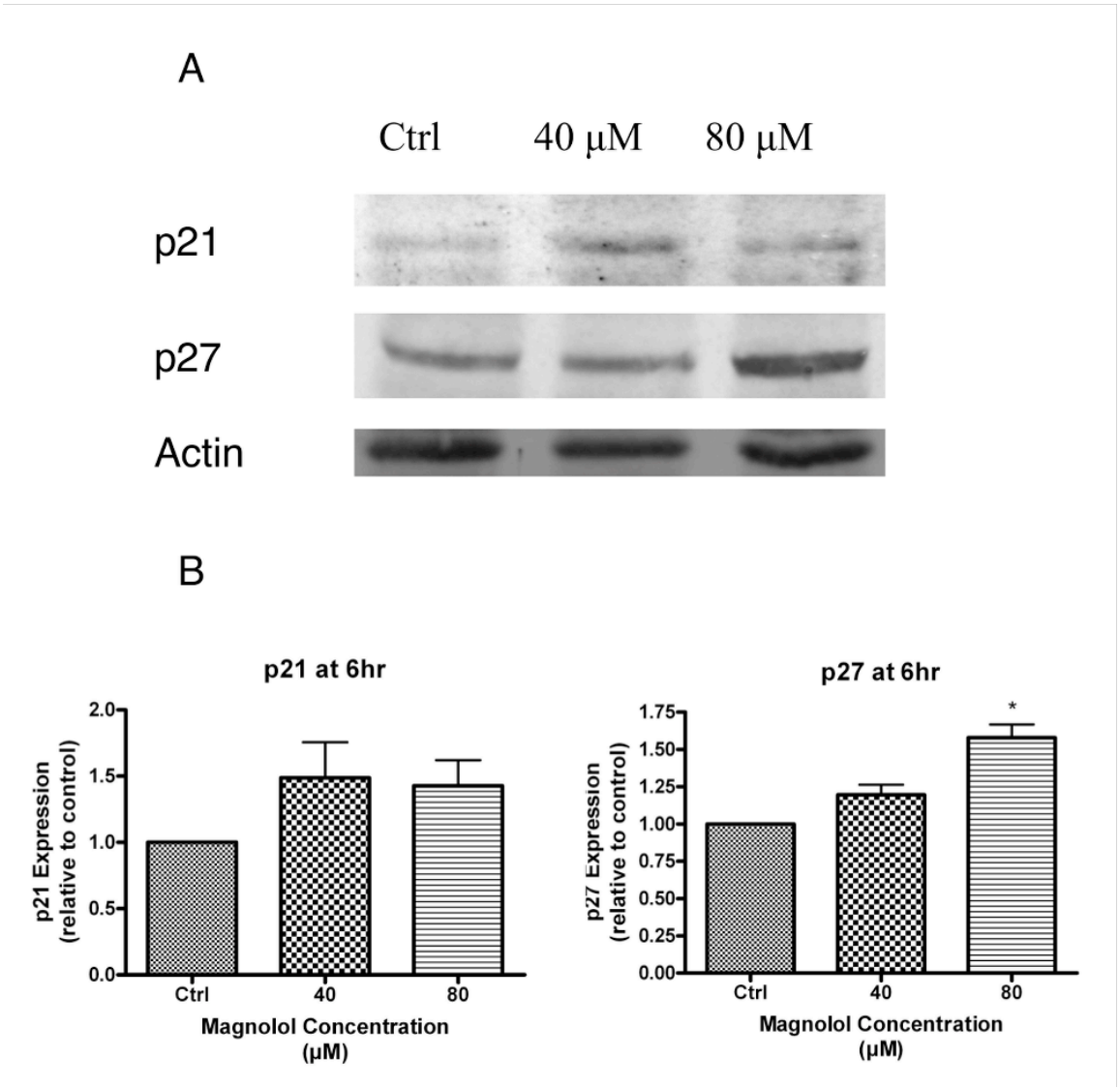

Figure 2.10: Magnolol affects expression of p21 and p27 after 6 h in PC3 cells *in vitro*. Cells were treated with either DMSO (Ctrl), 40 or 80 μM magnolol in DMSO as indicated for 6 h. Actin was used as a loading control. Each blot shown is representative of results obtained from at least three replications using separate samples. (*) indicates statistical significance at $P \leq 0.05$.

unchanged at 40 μM exposure to magnolol after 6 h in both DU145 and PC3 cells, but increased by 34% and 58% after exposure to 80 μM magnolol for 6 h in DU145 and PC3 cells, respectively.

2.4 Discussion

In this study, the effect of magnolol on the behavior of DU145 and PC3 human prostate cancer cells *in vitro* was examined. Specifically, magnolol was found to be cytotoxic to cells and to elicit alterations in the cell cycle, thereby preventing the cells from entering the G_2/M-phase. Associated with these cell cycle alterations, protein expression of several key cell cycle proteins was affected. The concentrations of magnolol used in the present study were 40 and 80 μM for 24 h, similar to those concentrations used in previous studies on the effects of magnolol on various cancer cell lines *in vitro* (Chuang *et al*. 2011; Hsu *et al*. 2007; Hwang and Park 2010; Lee *et al*. 2008a). The lower concentration used in this study, 40 μM, is equivalent to the highest tested concentration of magnolol measured in the blood plasma of rats injected with 10 mg/kg magnolol in a previous study (Tsai *et al*. 1996). This concentration can therefore be reasonably achieved in *in vivo* models. The *in vitro* cytotoxicity observations in the present study confirm previous *in vitro* cytotoxicity work using DU145 and PC3 cells exposed to magnolol by Lee *et al*. (2009) upon which the concentrations used in this study were based. This basis allowed this study to show, for the first time, that magnolol can cause alterations to the cell cycle in human prostate cancer cells and that expression of cell cycle proteins can be modulated by magnolol.

One of the hallmarks of cancer is dysregulation of the cell cycle leading to unmitigated cell growth and division (Hanahan and Weinberg 2011). The present study is consistent with other literature showing that magnolol can cause alterations in the cell cycle in cancerous cells *in vitro*. Flow cytometric analysis showed that DU145 and PC3

cells underwent cell cycle changes in response to 40 and 80 μM magnolol exposure after 24 h; both DU145 and PC3 cells showed an increased proportion of cells in the G_0/G_1-phase of the cell cycle and a decreased proportion in the G_2/M-phase. A G_0/G_1 arrest in response to magnolol exposure is observed in human colon and liver cancer cells (Lin *et al*. 2002) and in human glioblastoma cells (Chen *et al*. 2009). However, a G_2/M-phase arrest of the cell cycle was observed in human urinary bladder cells (Lee *et al*. 2008a) and cell cycle arrest in the S-phase was found in human gastric cancer cells (Rasul *et al*. 2012). Honokiol, a structural isomer of magnolol, has also previously been found to cause cell cycle arrest in the G_0/G_1-phase of human prostate cancer cells (Hahm and Singh 2007) and human breast cancer cells (Wolf *et al*. 2007). From this it appears that the cellular response to magnolol differs in cells from different organs or tissues of origin, with various cell types showing cell cycle arrest in different phases of the cell cycle.

Associated with changes to the cell cycle are changes in expression of key cell cycle proteins. Schwartz and Shah (2005) provide an overview of the cell cycle, describing the function of each cyclin and CDK protein: cyclin D1 acts with CDK2, CDK4 and CDK6 to progress the cell through the G_1-phase, cyclin E acts in conjunction with CDK2 to transition the cell into the S-phase, cyclin A and CDK2 progress the cell through the S-phase and cyclin B1 is involved in mitosis. Alterations of the cell cycle resulting in a decreased number of cells entering the G_2/M-phase would therefore necessitate interruption of the expression of cyclin D1, cyclin E, CDK2 or CDK4; all 4 of which show decreased expression in the presence of magnolol in DU145 cells and two of which (cyclin D1 and CDK2) show decreased expression in the presence of magnolol in

PC3 cells. This is consistent with other studies showing a G_0/G_1-phase arrest in human cancer cells. Chen *et al*. (2009) observed a decrease in cyclin D1 and cyclin A in human glioblastoma cells exposed to magnolol *in vitro*. Lin *et al*. (2002) also observed a decrease in cyclin A, cyclin E and CDK2 in human colon cancer cells exposed to magnolol *in vitro*. When PC3 cells were exposed to honokiol by Hahm and Singh (2007), decreased expression of cyclin D1, cyclin E, CDK2 and CDK4 was observed. The similarities between the two compounds, magnolol and honokiol, as well as the use of the same cell line suggests that similar results should be expected in the present study and this is what was observed. This study shows, for the first time, that magnolol affects the protein expression of cell cycle inhibitors at leading to alterations of the cell cycle at later time points.

The expression of these cyclins and CDKs are controlled by several inhibitor proteins which may also have been affected by magnolol exposure. To this end, protein expression of several key cell cycle inhibitor proteins (including $p16^{INK4a}$, p21, p27, pRBp107 and pRBp130) was determined by Western blot analysis. No significant changes were observed in the expression of $p16^{INK4a}$, p21 or p27 after 24 h exposure to either 40 or 80 μM magnolol. This was unexpected as an increase in the expression of these proteins would lead to a decrease in many of the cyclins and CDKs: particularly, $p16^{INK4a}$ inhibits CDK4 which is responsible for the transition from G_1 to S-phase; p21 and p27 broadly inhibit CDKs and can prevent CDK-cyclin complexes from forming, thereby preventing progression through the cell cycle as well (Johnson and Walker 1999; Vermeulen *et al*. 2003). In their examination of the effects of honokiol on human prostate

cancer cells *in vitro*, Hahm and Singh (2007) found that expression of p21 increased in PC3 cells exposed to honokiol up to 8 h of exposure and subsequently decreased to basal levels thereafter, returning to levels comparable to the control by 24 h. As a result, protein expression of p21 and p27 was examined in DU145 and PC3 cells after 6 h exposure to magnolol, where both proteins trended toward increased expression with p27 showing a significant increase at 80 μM at the 6 h time point in both cell lines. Eto (2013) found that p27 expression was inversely related to carcinogenic risk *in vivo* in mouse models, concluding that a loss of p27 increased the chance of these mice developing cancer. Similarly to the present study, Lee *et al*. (2008a) observed an increase in p27 expression and no change in p21 expression in human urinary bladder cancer cells *in vitro* after exposure to magnolol. Colon cancer and glioblastoma cells exposed to magnolol increased p21 expression with no observed change in p27 expression (Lin *et al*. 2002; Rasul *et al*. 2012).

Finally, pRBp107 and pRBp130 protein expression levels were examined by Western blot analysis to determine if altered expression of these proteins was involved in the cellular response to magnolol. Expression of pRBp107 protein level was significantly reduced at both 40 and 80 μM magnolol in both DU145 and PC3 cells while pRBp130 protein expression level exhibited a significant increase at 40 μM in DU145 cells. Only a nonsignificant trend toward increased protein expression of pRBp130 protein was observed at the higher, 80 μM, concentration. The pRB proteins are also involved in the regulation of the cell cycle, with pRBp107 and pRBp130 typically mediating the inhibitory effects of $p16^{INK4a}$ (Leng *et al*. 2002). In this sense, cells lacking pRBp107 or

pRBp130 did not undergo the cell cycle arrest typical of increased $p16^{INK4a}$ expression (Bruce *et al*. 2000). As no significant change was observed in $p16^{INK4a}$ expression in the present study, the decreased pRBp107 protein expression would likely not play a role in the observed changes to the cell cycle through this pathway. The E2F family of proteins are associated with progression from G_1 to S-phase of the cell cycle and pRBp130 is known to bind these E2F proteins and thereby stop cell cycle progression (Mayol and Graña 1997). As increased expression of pRBp130 protein was observed in the present study, this could be one possible explanation for the observed cell cycle alterations. It has also been suggested that differential regulation of pRBp130 and pRBp107, consisting of increased pRBp130 expression and decreased pRBp107 expression, is associated with exit from the cell cycle in serum dependent cells subsequently deprived of serum; the opposite was found to be true in cells transitioning from G_1 to S-phase where pRBp130 expression was lowered and pRBp107 expression increased (Mayol and Graña 1997; Smith *et al*. 1996).

In conclusion, this study has demonstrated that magnolol is able to induce alterations in the cell cycle resulting in a decreased number of cells entering the G_2/M-phase of the cell cycle in the DU145 and PC3 androgen insensitive human prostate cancer cell lines *in vitro*. These cell cycle alterations are associated with changes in the expression of key cell cycle regulatory proteins, most notably cyclins A, B1, D1 and E, CDK 2 and CDK4, p27 and pRBp130. In completing this study the anti-cancer properties of magnolol *in vitro* are further elucidated and contribute to the body of evidence suggesting that magnolol has potential as a novel anti-prostate cancer agent. This study

highlights the role of magnolol in altering the cell cycle of human prostate cancer cells *in vitro* through modulation of cell cycle protein expression as well as alterations to the protein expression of cell cycle inhibitors. Further research is required to determine if the effects observed in the present study also occur in androgen sensitive prostate cancer cell lines, such as LNCaPs, and to determine if the *in vitro* effects observed in the present study are replicable in an *in vivo* model.

CHAPTER THREE

Magnolol Affects Cellular Proliferation, Polyamine Biosynthesis and Catabolism-Linked Protein Expression and Associated Cellular Signaling Pathways in Human Prostate Cancer Cells *In Vitro*[2]

3.1 Introduction

Prostate cancer is the most commonly diagnosed form of cancer in men in Canada and the United States, representing roughly 10% of cancer related deaths in men in both countries (ACS 2011; CCS 2013). As a result there is an increased interest in the research of natural products, many of which have traditionally been used in herbal medicines, and their bioactive effects and potential chemoprotective and chemopreventative capacities (Bemis *et al*. 2006; Ikarashi *et al*. 2001; Zhang *et al*. 2005).

Magnolol, a lignan found in the roots and bark of the magnolia tree *Magnolia officinalis*, has previously been shown to affect important cellular processes such as the cell cycle and apoptosis in a variety of human cancer cell types both *in vitro* and *in vivo* (Chuang *et al*. 2011; Hsu *et al*. 2007; Hwang and Park 2010; Kim *et al*. 2007; Lee *et al*. 2009; Lee *et al*. 2008a; Lin *et al*. 2001, 2002; Rasul *et al*. 2012). Those studies performed *in vivo* suggest that magnolol remains active when administered at a sufficient oral dosage. Animal models have been used to determine the bioavailability of magnolol using two delivery methods: oral administration and intravenous injection. It was found

[2] The results of this study have been previously published in the Journal of Functional Foods in Health and Disease (McKeown and Hurta 2015). This chapter is an adaptation and reformatting of that publication.

that an injected dose of 2-10 mg/kg could achieve a maximum blood plasma concentration of 10 μg/ml or 40 μM magnolol while oral administration of 20 mg/kg could achieve a maximum blood plasma concentration of 0.1 μg/ml or 0.4 μM magnolol (Ho and Hong 2012; Lin *et al*. 2011; Tsai *et al*. 1996). Magnolol has also been shown to be non-toxic and to have no drug related side effects at any dose including the highest tested doses in several studies (Ho and Hong 2012; Li *et al*. 2007; Liu *et al*. 2007). These previously observed effects warrant further investigation into the mechanisms by which the cell responds to the anti-cancer effects of magnolol.

Polyamines (putrescine, spermidine and spermine) are essential to various cellular processes, particularly cellular growth, and the levels of polyamines are carefully controlled within a narrow range (Perez-Leal and Merali 2012). These compounds can bind to RNA, DNA and proteins to change their functions or to increase their expression; one of the most common ways in which polyamines affect cells is in their binding to mRNA and stimulating protein synthesis by causing structural changes (Igarashi and Kashiwagi 2009). The biosynthesis of polyamines is controlled by ornithine decarboxylase (ODC), a rate limiting factor in the conversion of ornithine to putrescine, S-adenosylmethionine decarboxylase (SAMDC) is then responsible for further biosynthesis from putrescine to spermidine and from spermidine to spermine. Polyamine catabolism is controlled by two further proteins, polyamine oxidase (PAO) and spermidine/spermine N1-acetyltransferase (SSAT), both of which are capable of converting spermine to spermidine and spermidine to putrescine. Elevated polyamine levels have been associated with prostate cancer and altered expression of the rate

limiting enzymes involved in both polyamine biosynthesis and catabolism are often observed during the development of cancer (Nowotarski *et al*. 2013). In particular, ODC expression is generally increased in human cancers and therapies targeting these polyamine biosynthetic/catabolic enzymes have been used in both chemotherapy and in chemoprevention (Nowotarski *et al*. 2013).

In the present study, the *in vitro* effects of magnolol on the expression of proteins involved in the biosynthesis and catabolism of polyamines in PC3 human prostate cancer cells were examined. This is novel research as the effects of magnolol on this polyamine pathway have not yet been investigated. Furthermore this investigation seeks to determine the degree to which polyamine related proteins are modulated in response to magnolol exposure and to characterize the cellular signaling mechanisms involved in the effect of magnolol on cellular proliferation.

3.2 Materials and Methods

All antibodies used in this study were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). These include: goat polyclonal anti-ODC, anti-AZ, anti-R1, anti-R2, anti-p-JNK, anti-JNK-1, anti-p-PI3Kp85, anti-Akt, anti-Histone H2B and anti-p-IκBα; mouse polyclonal anti-actin, anti-PAO, anti-SAMDC, anti-PI3Kp85 and anti-IκBα; and rabbit polyclonal anti-SSAT, anti-AZI, anti-p-ERK, anti-ERK-1, anti-ERK-2, anti-p-p38, anti-p38, anti-JNK-2, anti-PI3Kp110, anti-p-Akt, anti-NFκBp65, anti-c-jun and anti-c-fos. Magnolol was purchased from LKT Laboratories, Inc. (St. Paul, MN). All other chemicals and materials were purchased from Sigma-Aldrich Canada (Oakville, ON) unless otherwise indicated.

3.2.1 Cell Culture and Treatment with Magnolol

Human PC3 prostate adenocarcinoma cells (ATCC, Manasses, VA) were cultured on 100 mm plastic tissue-culture dishes (Falcon, Mississauga, ON) in RPMI (Gibco, Burlington, ON) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Invitrogen Canada, Burlington, ON), and were incubated at 37 ^{0}C in 5% CO_2. At 70% confluence, cells were exposed to 80 μM concentrations of magnolol, dissolved in dimethyl sulfoxide (DMSO), for 6 h [concentration of DMSO in both magnolol-treated and control cells was 0.2% (v/v)]. Control cells received only DMSO. After treatment, cells were removed using trypsin diluted in phosphate buffered saline (PBS) and re-suspended in RPMI with 10% FBS and centrifuged for 5 min at 500x G. The medium was then removed by aspiration. The remaining cell pellet was re-suspended and washed

in cold PBS. Cells were again centrifuged for 5 min at 500x G. After centrifugation, the PBS was removed by aspiration and the pellet was then stored at -80 ^{0}C until further analysis.

3.2.2 Cellular Proliferation Curve

PC3 human prostate adenocarcinoma cells were subcultured at 25000 cells/plate on 100 mm plastic tissue culture dishes in RPMI supplemented with 10% FBS and 1% antibiotic-antimycotic. Following 24 h of incubation, cells were counted using a haemocytometer to determine cell number at the 0 h time point. Cells were then treated with either 80 μM magnolol in DMSO or DMSO alone (control). Cells were again counted at 6, 24, 48 and 72 h time points. Triplicate plates were counted for each treatment (control or 80 μM magnolol) at each time point.

3.2.3 Nuclear and Cytosolic Protein Fraction Isolation

Nuclear and cytosolic protein isolation was performed using a nuclear extraction kit (Cayman Chemical, Ann Arbor, MI) as per manufacturer's instruction. Briefly, cells were treated and pelleted as above then cell pellets were suspended in 250 μL of hypotonic buffer (10% 10x hypotonic buffer, 2% phosphatase inhibitors, 1% protease inhibitors, 87% distilled water) and incubated on ice for 15 min. After 15 min, 50 μL of 10% Nonident P-40 assay reagent was added to each sample and mixed by pipetting. Samples were centrifuged at 14000x G for 30 sec at 4 ^{0}C. The supernatant was then removed and stored at -80 ^{0}C as this represents the cytosolic fraction. The remaining cell pellet was

resuspended in 50 µL of extraction buffer (50% 2x nuclear extraction buffer, 2% phosphatase inhibitors, 1% protease inhibitors, 37% distilled water) and vortexed for 15 sec, then shaken for 15 min at 0 ^{0}C. Samples were vortexed, for 30 sec, and shaken a second time for 15 min at 0 ^{0}C. Finally, samples were centrifuged at 14000x G for 10 min at 4 ^{0}C and the supernatant, representing the nuclear fraction, was removed and stored at -80 ^{0}C until further immunoblot analysis.

3.2.4 Immunoblot Analysis

Cell pellets (for those sample not undergoing nuclear isolation) were re-suspended in 100 µl of 10 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM PMSF, and briefly sonicated. Cell lysates were then centrifuged at 9300x G for 20 min at 4 ^{0}C. The supernatant was removed from the pellet as this represents a whole cell lysate sample. Nuclear, cytosolic and whole lysate samples were evaluated for protein content. Equal amounts of protein from these extracts were mixed in a 3:1 ratio with standard Laemmli buffer consisting of 50 mM Tris-HCl (pH 6.8), 10% SDS, 0.1% bromophenol blue, 10% glycerol and 100 mM β-mercaptoethanol and boiled for 3 min. Electrophoresis through 10% SDS-PAGE gels was used to resolve proteins which were then transferred onto nitrocellulose membranes (Bio-Rad, Mississauga, ON) by electro-blotting. Membranes were then incubated in the presence of a 1% BSA (w/v) TBS-Tween (0.05% v/v) solution overnight at 4 ^{0}C. Primary antibodies diluted to 1:200 (v/v) were then applied to membranes and incubated for 1 h at room temperature. After incubation, the membranes were washed three times with TBS-Tween (0.05% v/v) for a total of 30 min then incubated with

alkaline phosphatase (AP)-conjugated secondary antibodies (1:1000) for 1.25 h at room temperature. After incubation the membranes were again washed three times with TBS-Tween (0.05%) for a total of 30 min and rinsed quickly with distilled water, then exposed to SigmaFast BCIP-NBT tablets (Sigma-Aldrich, Oakville, ON) dissolved in distilled water to visualize protein expression levels. Western blots were then photographed using Infinity Capture Software (Lumenera Corp., Ottawa, ON) and densitometry was performed with ImageJ Software (National Institute of Health, Bethesda, MD).

3.2.5 Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 4.03 for Windows (GraphPad Software, Inc. San Diago, CA). Cell counts in cellular proliferation curve were compared to corresponding cell count for control cells at the appropriate time point using a simple t-test and results were considered statistically significant at $P \leq 0.1$. Results of immunoblot analysis were compared using a simple t-test and results were considered statistically significant at $P \leq 0.1$.

3.3 Results

3.3.1 Magnolol Affects Cellular Proliferation

It has previously been shown that magnolol is cytotoxic to PC3 human prostate cancer cells *in vitro*, as discussed in Chapter Two of this thesis (Lee *et al*. 2009; McKeown *et al*. 2014). To confirm that this cytotoxicity is accompanied by reduced cellular proliferation a cellular proliferation curve experiment was conducted (Figure 3.1). Time course experiments show that cellular proliferation is retarded in PC3 cells exposed to 80 μM magnolol. At 6 h exposure to magnolol there was no significant difference in cellular proliferation between magnolol exposed and control cells, but by 24 h a statistically significant difference in cell numbers was observed whereby control cell count increased to 231% of 0 h cell count and magnolol exposed cell count only increased to 151% of 0 h cell count. Cellular proliferation in magnolol exposed cells essentially stopped at 48 h where cell counts were 154% of 0 h counts, only a 2% increase from 24 h counts. By 72 h magnolol exposed cell counts were lower than the 0 h count while control cells counts were at 714% of 0 h counts.

3.3.2 Magnolol Affects Proliferation-Linked Protein Expression

Having established that magnolol exposure results in decreased cellular proliferation, expression of proteins involved in two important proliferation-linked activities were examined. In PC3 cells exposed to 80 μM magnolol for 6 h the protein expression of ornithine decarboxylase (ODC), antizyme inhibitor (AZI), spermidine/spermine N1-acetyltransferase (SSAT) and polyamine oxidase (PAO) decreased significantly after

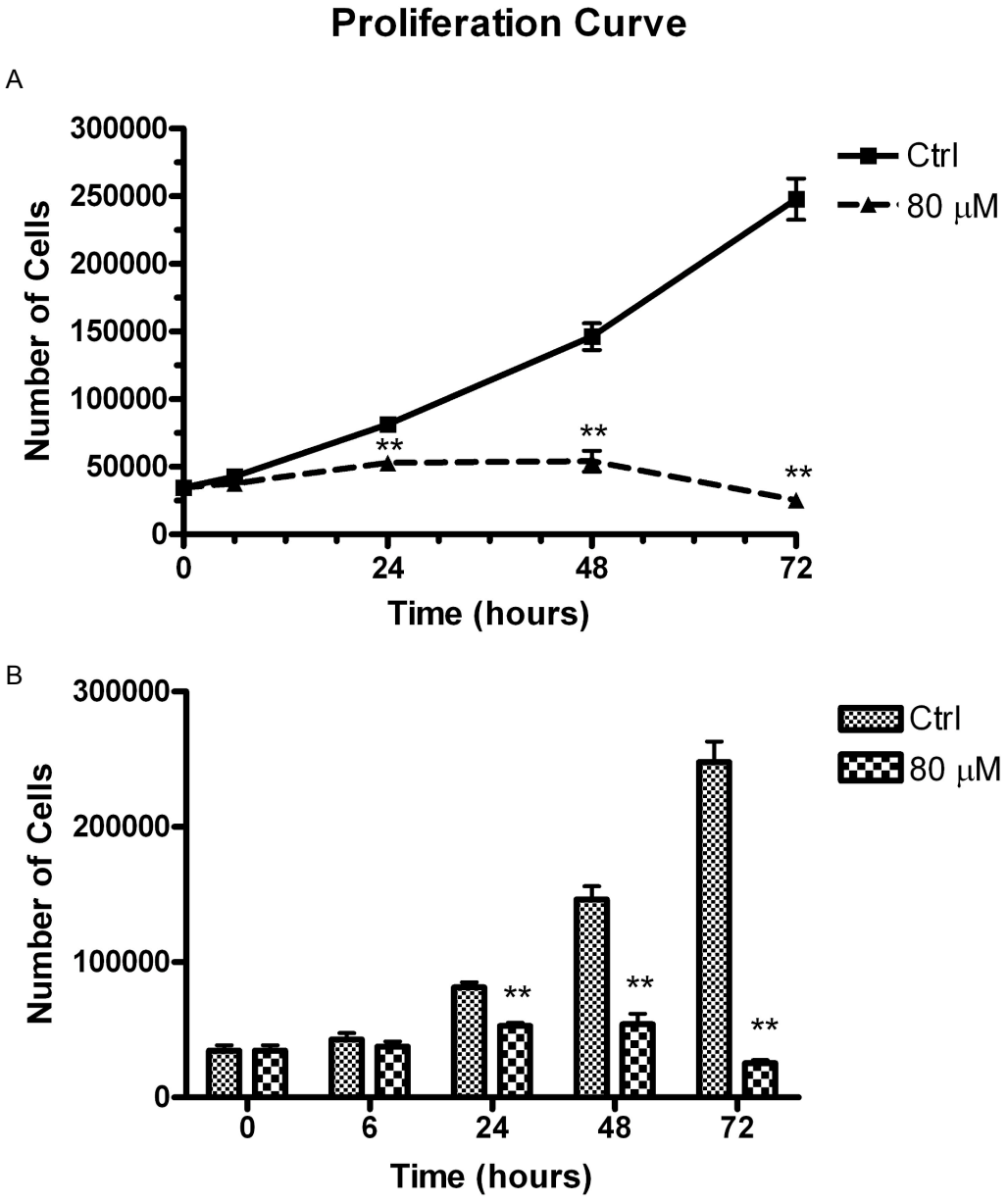

Figure 3.1: Magnolol affects cellular proliferation of PC3 human prostate cancer cells *in vitro*. Both a line graph (A) and a bar graph (B) of this data set is presented for comparative purposes. Cells were treated with either DMSO (Ctrl) or 80 μM magnolol in DMSO for 6, 24, 48 or 72 h. The number of cells present under these conditions were determined in three separate experiments for each time point. Statistical significance between control and magnolol treated cells was determined at each time point. (*) indicates statistical significance at $P \leq 0.1$ and (**) indicates statistical significance at $P \leq 0.05$.

exposure to magnolol while protein expression of S-adenosylmethionine decarboxylase (SAMDC) increased (Figure 3.2). Antizyme (AZ) protein expression remained unchanged (Figure 3.2). While ODC protein expression decreased to 68% of control and AZI protein expression decreased to 73% of control, protein expression of SAMDC increased to 154%. Of those proteins directly involved in polyamine catabolism, SSAT protein expression decreased to 66% of control and PAO protein expression decreased to 43% of control.

To further determine the effect of magnolol on cellular proliferation in PC3 cells the two subunits of ribonucleotide reductase (R1 and R2) were assayed (Figure 3.3). As ribonucleotide reductase is necessary for the synthesis of deoxyribonucleoside triphosphates, the building material for DNA, changes in protein expression of the R1 or R2 subunits would represent an important pathway by which cellular proliferation may be affected (Guarino *et al*. 2014; Norlund and Reichard 2006). Ribonucleotide reductase is tightly linked to DNA synthesis and changes to either subunit suggest similar changes to DNA synthesis. While R1 protein expression was not significantly affected by magnolol exposure the protein expression of R2 decreased to 62% of control.

3.3.3 Magnolol Affects Cellular Signaling Pathway Protein Expression

Following the changes observed in expression of polyamine biosynthesis and catabolism-linked proteins several cellular signaling pathways were assayed to determine which, if any, of these pathways may be involved in the cellular response to magnolol exposure.

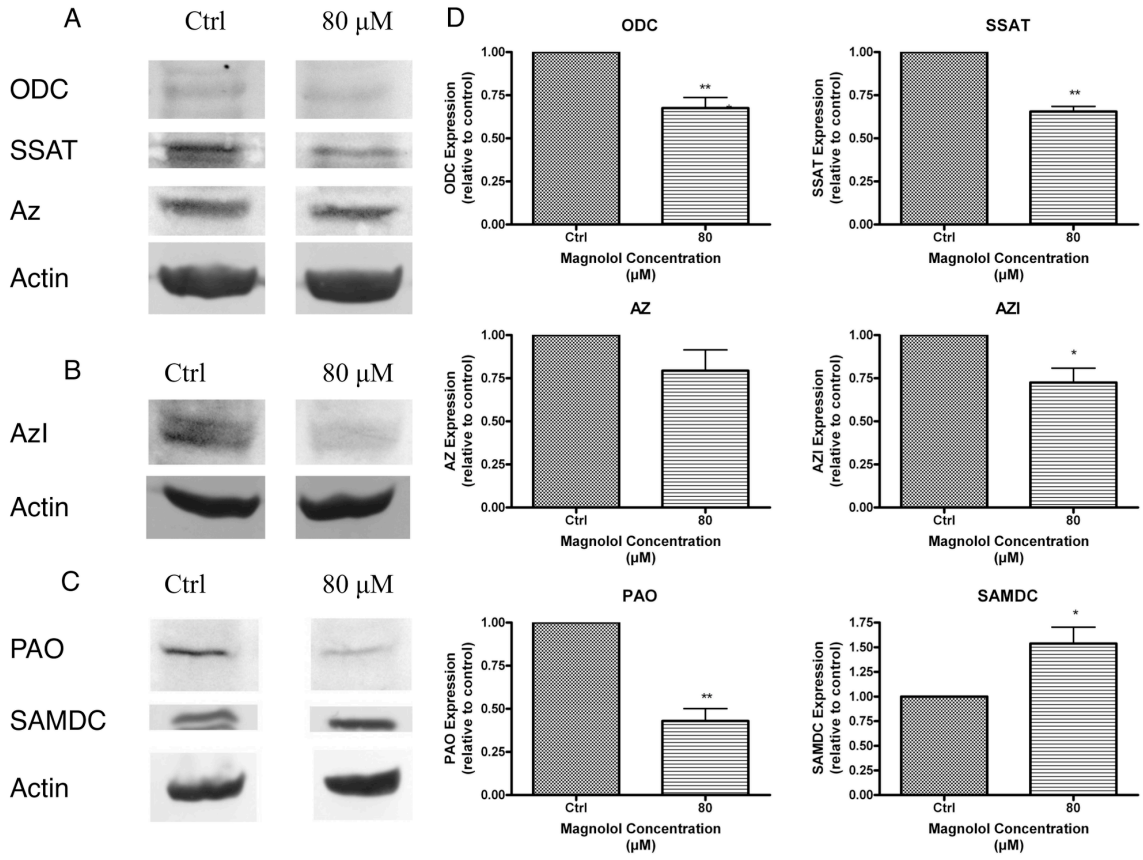

Figure 3.2: Magnolol affects expression of polyamine biosynthesis/catabolism-linked proteins in PC3 cells *in vitro*. Cells were treated with either DMSO (Ctrl) or 80 μM magnolol in DMSO for 6 h. Actin was used as a loading control (the actin used in Figure 3.2A is the same as those used in Figures 3.3B, 3.4B and 3.5B; the actin used in Figure 3.2B is the same as those used in Figures 3.3A and 3.5C as these samples were run concurrently). Each blot shown is representative of results obtained from at least three replications from three separate experiments. A, B and C: Representative Western blots of proteins involved in polyamine biosynthesis and catabolism. D: Graphical representation of protein expression. (*) indicates statistical significance at $P \leq 0.1$ and (**) indicates statistical significance at $P \leq 0.05$.

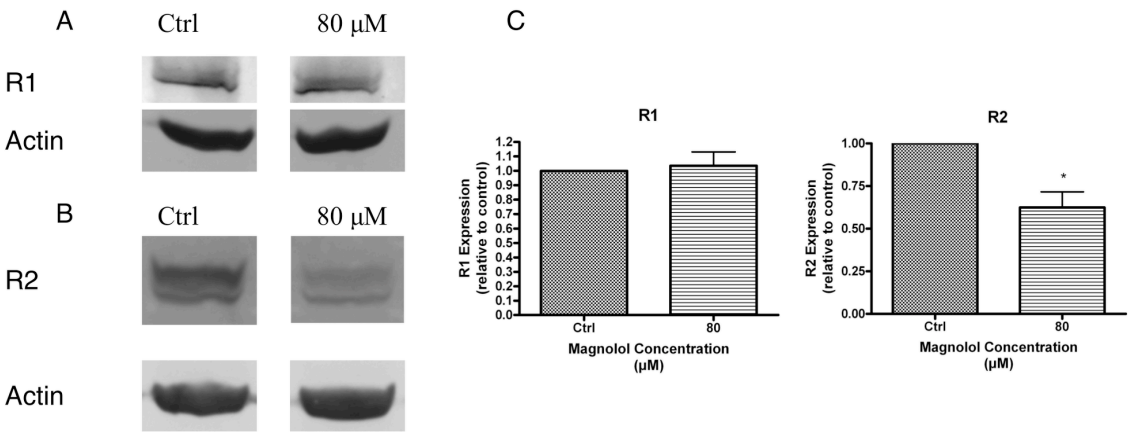

Figure 3.3: Magnolol affects expression of R2 subunit of ribonucleotide reductase in PC3 cells *in vitro*. Cells were treated with either DMSO (Ctrl) or 80 μM magnolol in DMSO for 6 h. Actin was used as a loading control (the actin used in Figure 3.3A is the same as those used in Figures 3.2B and 3.5C; the actin used in 3.3B is the same as those used in Figures 3.2A, 3.4B and 3.5B as these samples were run concurrently). Each blot shown is representative of results obtained from at least three replications from three separate experiments. A and B: Representative Western blots of the R1 and R2 proteins, respectively. C: Graphical representation of protein expression. (*) indicates statistical significance at $P \leq 0.1$.

By investigating these cellular signaling pathways and their regulators the signaling mechanism(s) through which magnolol acts can be further elucidated.

Of the MAPK pathways (Figure 3.4) proteins the expression of the following were significantly altered: p-p38 decreased to 75%, c-Jun N-terminal kinase (JNK)-2 decreased to 81%, JNK-1 decreased to 41% and p-JNK-1 increased to 116% (Figure 3.4). Of the PI3K pathway proteins (Figure 3.5) the expression of the following were significantly altered: PI3Kp85 decreased to 26%, p-PI3Kp85 decreased to 74% and p-Akt decreased to 63% (Figure 3.5). The expression of the following proteins of the NFκB pathway (Figure 3.6) were significantly altered from control: NFκBp65 nuclear fraction decreased to 60%, NFκBp65 cytosolic fraction decreased to 88%, p-IκBα decreased to 76% and nuclear factor of kappa-B inhibitor (IκBα) decreased to 84%. Finally, of the transcription factors involved in the activator protein-1 (AP-1) pathway (Figure 3.6) assayed, the expression of c-jun significantly increased to 207% while no statistically significant change was observed in expression of the c-fos protein.

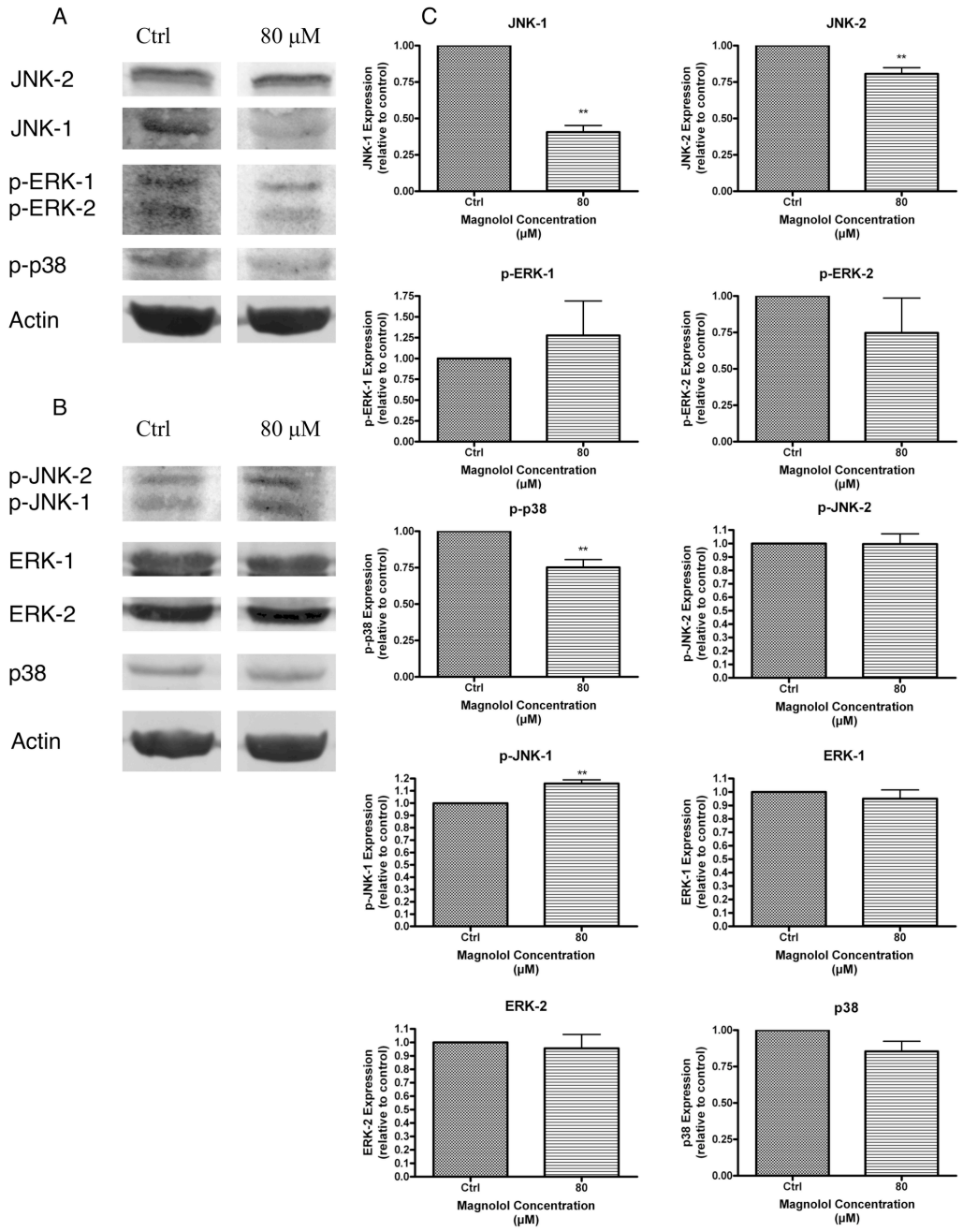

Figure 3.4: Magnolol affects expression of MAPK pathway proteins in PC3 cells *in vitro*. Cells were treated with either DMSO (Ctrl) or 80 μM magnolol in DMSO for 6 h. Actin was used as a loading control (the actin used in Figure 3.4A is the same as that used in Figure 3.5A; the actin used in 3.4B is the same as those used in Figures 3.2A, 3.3B and 3.5B as these samples were run concurrently). Each blot shown is representative of results obtained from at least three replications from three separate experiments. A and B: Representative Western blots of proteins involved in MAPK pathways. C: Graphical representation of protein expression. (**) indicates statistical significance at $P \leq 0.05$.

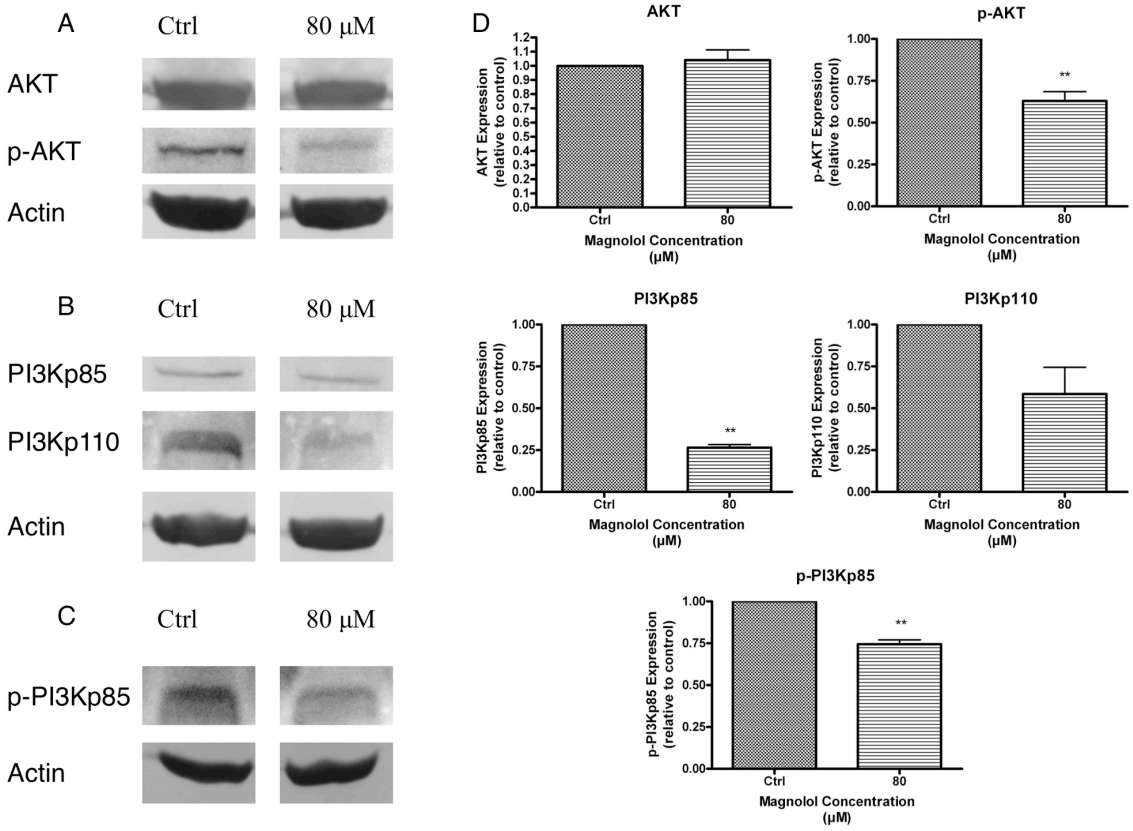

Figure 3.5: Magnolol affects expression of PI3K pathway proteins in PC3 cells *in vitro*. Cells were treated with either DMSO (Ctrl) or 80 μM magnolol in DMSO for 6 h. Actin was used as a loading control (the actin used in Figure 3.5A is the same as that used in Figure 3.4A; the actin used in 3.5B is the same as those used in Figures 3.2A, 3.3B and 3.4B as these samples were run concurrently). Each blot shown is representative of results obtained from at least three replications from three separate experiments. A: Representative Western blots of Akt and p-Akt proteins. B: Representative Western blots of PI3K proteins. C: Representative Western blot of phosphorylated PI3Kp85 protein. D: Graphical representation of protein expression. (**) indicates statistical significance at $P \leq 0.05$.

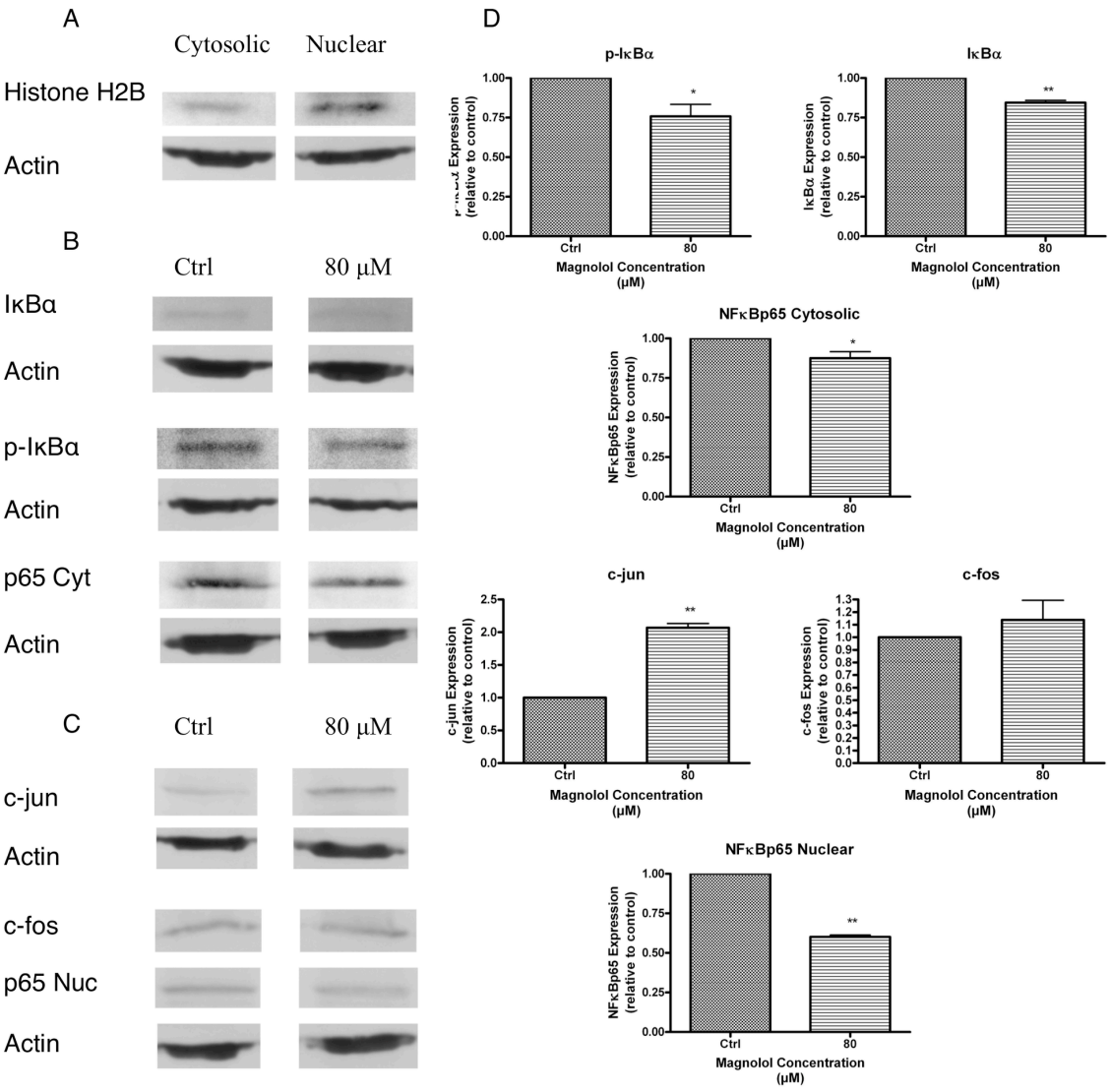

Figure 3.6: Magnolol affects expression of NFκB and AP-1 pathway proteins in PC3 cells *in vitro*. Cells were treated with either DMSO (Ctrl) or 80 μM magnolol in DMSO for 6 h. Actin was used as a loading control. Each blot shown is representative of results obtained from at least three replications from three separate experiments. A: Representative Western blot of histone H2B protein indicating nuclear protein enrichment. B: Representative Western blots of cytosolic fraction proteins of NFκB and AP-1 pathways. C: Representative Western blots of nuclear fraction proteins of NFκB and AP-1 pathways. D: Graphical representation of protein expression. (*) indicates statistical significance at $P \leq 0.1$ and (**) indicates statistical significance at $P \leq 0.05$.

3.4 Discussion

In this study the effect of magnolol on the behavior of PC3 human prostate cancer cells *in vitro* was examined. As discussed in Chapter Two of this thesis, magnolol has been found to cause alterations in expression of cell cycle proteins in DU145 and PC3 human prostate cancer cells (McKeown *et al* 2014). In the present study, magnolol was found to affect cellular proliferation through alterations to proteins involved in polyamine biosynthesis and catabolism. Cellular proliferation of PC3 cells exposed to magnolol was reduced after 24 h exposure and cell number remained virtually unchanged when cells were counted 48 h after exposure. Decreased protein expression of ODC in response to magnolol suggests that production of putrescine from ornithine is being limited. This is supported by reduced protein expression of AZI and unchanged protein expression of AZ. AZ acts as a non-competitive inhibitor of ODC, with AZ and ODC forming a dimer and increasing ODC degradation (Pegg 2006). By increasing protein expression of AZ in an *in vivo* mouse model of upper aerodigestive tract cancers, carcinogenesis was reduced and polyamine levels decreased (Feith *et al*. 2013). A reduction in AZI protein expression coupled with no significant change in AZ protein expression should similarly cause available AZ to be increased. Decreased ODC protein expression has also been associated with induction of apoptosis in MCF-7 breast cancer cells *in vitro* (Arisan *et al*. 2012). Magnolol has previously been shown by Lee *et al*. (2009) to induce apoptosis in PC3 human prostate cancer cells. The decreased protein expression of ODC observed in the present study combined with the finding of Lee *et al*. (2009) could indicate a similar connection between decreased ODC expression and apoptosis.

SAMDC functions in production of spermidine from putrescine and spermine from spermidine. The increase in SAMDC protein expression is opposite to that of ODC suggesting that SAMDC may be compensating for the loss of ODC in driving polyamine production forward. In *in vivo* mouse models, an increase in SSAT protein expression was accompanied by increasing ODC protein expression, compensating for the increased polyamine catabolism by increasing biosynthesis (Kee *et al*. 2004; Tucker *et al*. 2005). This suggests that the decreases in SSAT and PAO in the present study may be due to a similar compensatory mechanism whereby decreased polyamine biosynthesis through reduced ODC protein expression is compensated with reduced SSAT and PAO protein expression resulting in reduced polyamine catabolism. Inhibition of PAO has also previously been linked to induction of apoptosis in certain leukemia cell lines *in vitro* (Wallace and Fraser 2004).

In assaying the protein expression of the R1 and R2 subunits of ribonucleotide reductase, only the protein expression of the R2 subunit decreased. As ribonucleotide reductase functions in converting ribonucleosides to deoxyribonucleosides, this enzyme is of key importance to cellular proliferation. R1 protein is constitutively expressed throughout the cell cycle, but R2 protein expression is most closely associated with the S-phase of the cell cycle (Guarino *et al*. 2014; Norlund and Reichard 2006). The change in the availability of the R2 subunit is due both to changes in protein expression as well as changes in protein degradation (Guarino *et al*. 2014). The R2 subunit is also recognized as the rate-limiting factor in conversion of ribonucleosides to deoxyribonucleosides, thereby acting as an important regulator of DNA synthesis with inhibition of this R2

subunit slowing down DNA synthesis (Guarino *et al*. 2014). As a result of its intimate ties to DNA synthesis, ribonucleotide reductase has been the target of chemotherapies in various types of cancer (Guarino *et al*. 2014). As magnolol has previously been shown to cause alterations in the cell cycle of PC3 human prostate cancer cells *in vitro*, the reduction of R2 protein expression in the current study further supports reduced proliferation and reduced ability for PC3 cells to proceed through the cell cycle in the presence of magnolol (McKeown *et al*. 2014).

Finally, several cellular signaling pathways were assayed to determine which, if any, are affected in the cellular response to magnolol exposure. In the MAPK pathways, magnolol has previously been shown to decrease protein expression of p38 and increase extracellular signal-regulated kinase p-(ERK)-1/2 protein expression with no significant change to protein expression of ERK-1/2 nor p-p38 in SKOV3 human ovarian cancer cells *in vitro* (Chuang *et al*. 2011). In COLO-205 human colon cancer cells *in vitro* magnolol exposure was associated with an increase in p-ERK-1/2 protein expression but protein expression of ERK-1/2 was not affected (Hsu *et al*. 2007). In human urinary bladder cancer 5637 cells *in vitro* exposed to 60 μM magnolol for 3, 6 and 12 h an increase in protein expression of p-ERK-1/2, p-p38 and p-JNK-1/2 with no apparent change in the non-phosphorylated forms occurred (Lee *et al*. 2008a). In U373 human malignant glioblastoma cells *in vitro* exposed to magnolol, increased protein expression of p-ERK-1/2 and p-p38 were observed after exposure to 100 μM magnolol for 60 min with no change to JNK-1/2, ERK-1/2, p38 or p-JNK-1/2 (Chen and Lee 2013). In VSMC human aortic smooth muscle cells *in vitro* no effect on ERK-1/2 or p-ERK-1/2 was

observed in response to magnolol (Kim *et al*. 2007). In the present study, the alterations to the MAPK pathways are relatively minor, with significant changes only occurring in p-p38, JNK-1/2 and p-JNK-1. The changes to MAPK protein expression are variable throughout these previous studies with no observed change to ERK-1/2 observed in any of the referenced studies and increases in protein expression of p-ERK-1/2 occurring in all studies aside from the present investigation and that of Kim *et al*. (2007). Inhibition of ERK-1/2 and p-ERK-1/2 has previously been associated with decreased expression of ODC in human leukemia L1210 cells *in vitro*, and increased ERK-1/2 and p-ERK-1/2 has associated with increased ODC. As no change in expression of these proteins was observed in the present study it is unlikely that they are involved in the decrease of ODC expression in response to magnolol observed (Flamigni *et al*. 1999). Protein expression of p-p38 is especially variable with increases in expression observed by Chen and Lee (2013) and Lee *et al*. (2008a), no change observed by Chuang *et al*. (2011) and a decrease in expression observed in the present study. The highly variable effect of magnolol on proteins of the MAPK pathways are likely due to these proteins being differently expressed across various cell lines.

Unlike the variable nature of the effect of magnolol on the MAPK pathways, the effects of magnolol on the expression of those proteins involved in the PI3K/Akt pathway appear to be conserved across cell lines. The PI3K/Akt signaling pathway has previously been found to be involved in apoptosis-related signaling in various cancer cell lines *in vitro* in response to magnolol exposure (Chuang *et al*. 2011; Lee *et al*. 2009; Rasul *et al*. 2012). These studies observed decreased protein expression of p-PI3K and p-Akt with no

change in protein expression of Akt. The results of the present study support those observations of a previous study by Lee *et al*. involving PC3 human prostate cancer cells in that alterations to cellular viability were associated with these changes to the PI3K pathway, but show an additional change in PI3Kp85 which was not observed previously (2009). This change may be related to the higher concentration of magnolol, 80 μM as opposed to 60 μM, used in the present study. While the PI3K pathway is typically associated with cell survival it also plays an important role in activating proliferation by controlling cyclin D and p27 activity (Vivanco and Sawyers 2002). The PI3K pathway can also control cell growth through mTOR, with decreased PI3K expression resulting in decreased cellular growth though this pathway (Vivanco and Sawyers 2002). The decreased activation of the PI3K/Akt pathway observed in the present study may affect both cellular growth and proliferation though these mechanisms.

The protein expression of NFκBp65 decreased in the cytoplasm and within the nucleus in the present study. This suggests that there is reduced expression of this protein and that translocation of NFκBp65 into the nucleus is reduced in response to magnolol exposure. NFκB is an inhibitor of apoptosis as well as being involved in the transcription, and activation, of cyclin D (Karin *et al*. 2002). Lastly, the increased protein expression of c-jun observed in the present study is contrary to the anti-proliferative effect of magnolol on PC3 human prostate cancer cells. As increased c-jun transcription is generally associated with increased cellular proliferation, it is possible that protein expression of c-jun is increased in a compensatory manner. While protein expression of c-fos was not significantly altered in response to magnolol exposure in the present study the increased

expression of c-jun may act in concert with stable expression of c-fos to activate the AP-1 pathway. While reduced expression of c-jun and c-fos is associated with decreased cellular proliferation, increased expression of these two proteins involved in the AP-1 pathway can also be an indication of apoptotic conditions although it is unknown if there is a functional role played by the AP-1 pathway or if it is merely an associated indicator (Shaulian and Karin 2002).

In conclusion, the present study has shown for the first time that magnolol does affect expression of proteins involved in cellular proliferation-linked activities. These alterations to the expression of proteins involved in cellular proliferation occurred in PC3 human prostate cancer cells *in vitro*. These effects were modulated through alterations in multiple cellular signaling pathways. This study is also novel in representing the most thorough investigation of the effects of magnolol on the cellular signaling pathways in human prostate cancer cells. Although the effect of magnolol on the MAPK signaling pathways are highly variable between various cancer cell types, the PI3K pathway is consistently altered and as such is a likely pathway by which magnolol affects cellular proliferation. Changes in the NFκB pathway similarly suggest that PC3 response to magnolol exposure is to reduce cellular proliferation. Further research is required to determine the effects of magnolol on cellular proliferation in an *in vivo* model, and to determine if magnolol affects cellular proliferation through alternative pathways such as disruptions to growth factor signaling.

CHAPTER FOUR

Magnolol Affects Expression of IGF-I and Associated Binding Proteins in Human Prostate Cancer Cells *In Vitro*[3]

4.1 Introduction

In Canada and the United States, prostate cancer is the most commonly diagnosed form of cancer among men and the second most common cancer among men worldwide (ACS 2011). This study continued previous investigation on the effects of magnolol, a compound found in the roots and bark of the magnolia tree *Magnolia officinalis*, by determining further pathways by which magnolol exposure may alter cellular proliferation. Specifically, this study investigated the effect of magnolol on insulin-like growth factor-I (IGF-I), the insulin-like growth factor-I receptor (IGF-IR) and the associated insulin-like growth factor binding proteins (IGFBPs) in PC3 and LNCaP human prostate cancer cells *in vitro*.

Insulin-like growth factors (IGFs) are involved in cellular growth, differentiation and apoptosis avoidance (Furlanetto *et al*. 1994; Yu and Rohan 2000). The cellular response to IGF is initiated through IGF binding to IGF-receptors (IGF-Rs). IGF action is regulated by IGFBPs, whether by increasing IGF half-life or by promoting/inhibiting IGF binding to IGF-Rs (Weinzimer *et al*. 2001). The IGFBPs function by having a greater binding affinity for IGF than do the IGF-Rs, thereby blocking the normal signaling

[3] The results of this study have been previously published in the journal Anticancer Research (McKeown and Hurta 2014). This chapter is an adaptation and reformatting of that publication.

interaction and allowing IGFBPs to functionally remove IGFs from the system in this way (Yu and Rohan 2000). IGFBPs also exhibit a range of IGF-independent activities: increased IGFBP-3 can result in reduced protein synthesis or induce apoptosis, while IGFBP-2 and IGFBP-5 can induce mitosis (Firth and Baxter 2002). Dramatic changes in IGFBP expression has also been associated with cancer, particularly in that IGFBP-2 and IGFBP-5 over-expression correlates with the change from androgen sensitivity to androgen independence in prostate cancer cells (DeGraff *et al*. 2007, 2009; Miyake *et al*. 2000). The present study examines the effect of magnolol on IGF-I and associated protein expression in two prostate cancer cell lines *in vitro*.

4.2 Materials and Methods

All antibodies used in this study were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibodies used included: mouse polyclonal anti-actin, anti-IGF-IR, anti-IGFBP-2 and anti-IGFBP-3; goat polyclonal anti-IGF-I; and rabbit polyclonal anti-p-IGF-IR, anti-IGFBP-4 and anti-IGFBP-5. Magnolol was purchased from LKT Laboratories, Inc. (St. Paul, MN). All other chemicals and materials were purchased from Sigma-Aldrich Canada (Oakville, ON) unless otherwise indicated.

4.2.1 Cell Culture and Treatment with Magnolol

Human PC3 prostate adenocarcinoma cells (ATCC, Manasses, VA) and human LNCaP prostate adenocarcinoma cells (ATCC) were cultured on 100 mm plastic tissue-culture dishes (Falcon, Mississauga, ON) in RPMI (Gibco, Burlington, ON) supplemented with

10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Gibco), and were incubated at 37 ^{0}C in 5% CO_2. After incubation, cells were exposed to 80 μM magnolol, dissolved in dimethyl sulfoxide (DMSO), for 6 h. Control cells received only DMSO. Magnolol (80 μM) was previously determined to be an effective concentration *via* cytotoxicity studies as discussed in Chapter Two of this thesis (McKeown *et al*. 2014). After treatment, cells were removed using trypsin diluted in phosphate buffered saline (PBS) and re-suspended in RPMI with 10% FBS and centrifuged for 5 min at 500x G. The medium was then aspirated off leaving only a cell pellet and the cells were resuspended and washed in cold PBS. Cells were again centrifuged for 5 min at 500Xg. After centrifugation, the PBS was aspirated off and the cell pellet was stored at -80 ^{0}C until further analysis.

4.2.2 Immunoblot Analysis

Cell pellets were resuspended in 100 μl of 10 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM PMSF, and briefly sonicated. Cell lysates were then centrifuged at 9300x G for 20 min at 4 ^{0}C. The supernatant was removed from the pellet and evaluated for protein content. Equal amounts of protein from this extract were mixed in a 3:1 ratio with standard Laemmli buffer consisting of 50 mM Tris-HCl (pH 6.8), 10% SDS, 0.1% bromophenol blue, 10% glycerol and 100 mM β-mercaptoethanol and boiled for 3 min. Electrophoresis through 10% SDS-PAGE gels was used to resolve proteins, which were then transferred onto nitrocellulose membranes (Bio-Rad, Mississauga, ON) by electroblotting. Membranes were then incubated in the presence of a 1% BSA (w/v) TBS-

Tween (0.05% v/v) solution overnight at 4 ^{0}C. Primary antibodies diluted to 1:200 (v/v) were then applied to membranes and incubated for 1 h at room temperature. After incubation, the membranes were washed 3 times with TBS-Tween (0.05% v/v) for a total of 30 min, then incubated with alkaline phosphatase (AP)-conjugated secondary antibodies (1:1000) for 1.25 h at room temperature. After incubation, the membranes were again washed 3 times with TBS-Tween (0.05%) for a total of 30 min and rinsed quickly with distilled water, then exposed to SigmaFast BCIP-NBT tablets (Sigma) dissolved in distilled water to visualize protein expression levels. Western blots were then photographed using Infinity Capture Software (Lumenera Corp., Ottawa, ON) and densitometry was performed with ImageJ Software (National Institute of Health, Bethesda, MD).

4.2.3 Statistical Analysis

Statistical analysis was conducted using Graphpad Prism 4.03 for Windows (Graphpad Software, Inc. San Diego, CA). Results of immunoblot analysis were compared a simple t-test and results were considered statistically significant at $P \leq 0.1$.

4.3 Results

4.3.1 Magnolol Affects IGF-I and IGFBP Expression

Protein expression of IGF-I, IGF-IR and IGFBPs were altered in PC3 (Figure 4.1) and in LNCaP (Figure 4.2) human prostate cancer cells after 6 h exposure to 80 μM magnolol. This magnolol exposure resulted in a reduction to 56% IGF-I in PC3 cells. Similarly, in LNCaP cells, magnolol exposure resulted in a significant IGF-I reduction to 70% of control. To determine if changes in IGF-I expression in response to magnolol were affected by changes in the IGFBPs, protein expression of IGFBP-2, IGFBP-3, IGFBP-4 and IGFBP-5 were also examined. Protein expression of IGFBP-2 was not significantly affected in either PC3 or LNCaP cells. IGFBP-3 protein expression increased significantly in both cell lines: to 132% in LNCaP cells and to 142% in PC3 cells. IGFBP-4 protein expression was not significantly affected by magnolol exposure in PC3 cells; however, expression was increased to 160% without attaining significance. In LNCaP cells IGFBP-4 protein expression was increased to 171%. The final IGFBP assayed, IGFBP-5, showed a significant decrease in protein expression in both LNCaP and PC3 cells in response to magnolol treatment at 79% and 68%, respectively. Finally, to determine if magnolol exposure would affect IGF signaling, the protein expression of IGF-IR and its phosphorylated form were examined. Neither IGF-IR nor p-IGF-IR were significantly affected by magnolol exposure in LNCaP cells; however, IGF-IR protein expression increased to 130% without attaining statistical significance. In PC3 cells the protein expression of IGF-IR increased to 132%, while the protein expression of p-IGF-IR decreased to 65%.

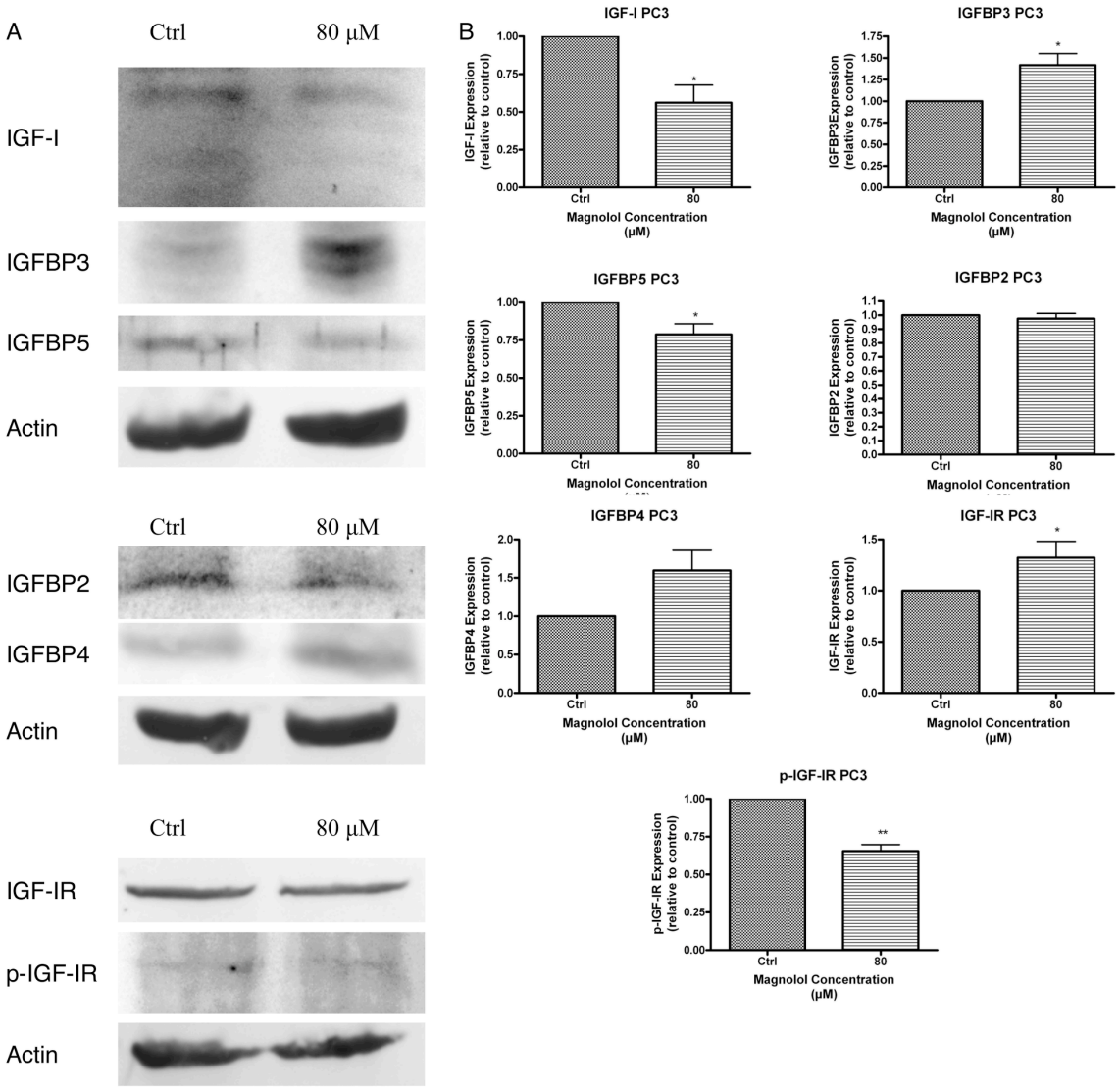

Figure 4.1: Magnolol affects expression of IGF-I and associated proteins in PC3 human prostate cancer cells *in vitro*. Cells were treated with either DMSO (Ctrl) or 80 μM magnolol in DMSO for 6 h. Actin was used as a loading control. Each blot shown in Figure 4.1A is representative of results obtained in triplicate using separate samples. These triplicate results are represented as changes in protein expression in the graphs in Figure 4.1B. (*) indicates statistical significance at $P \leq 0.1$ and (**) indicates statistical significance at $P \leq 0.05$.

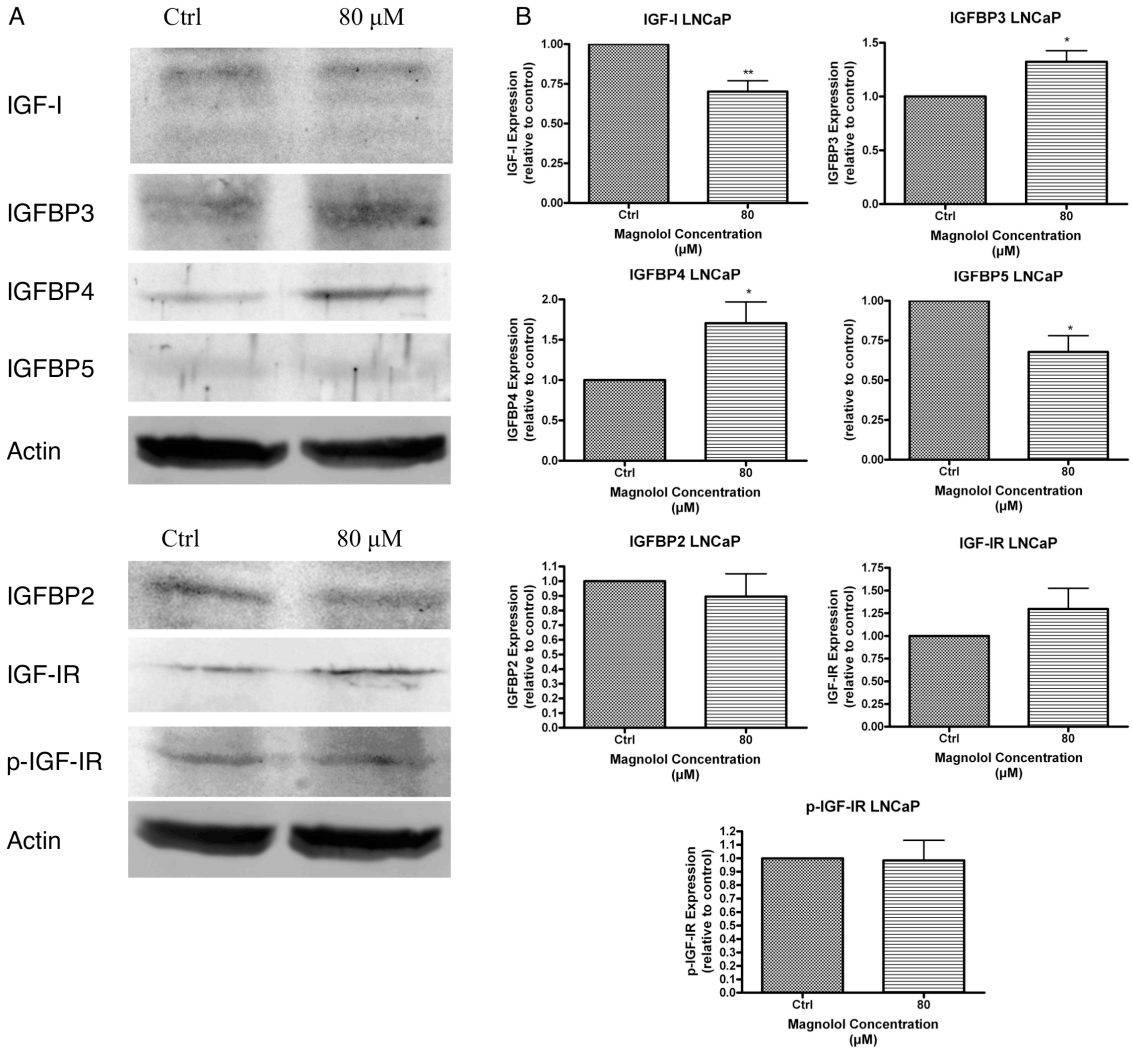

Figure 4.2: Magnolol affects expression of IGF-I and associated proteins in LNCaP human prostate cancer cells *in vitro*. Cells were treated with either DMSO (Ctrl) or 80 μM magnolol in DMSO for 6 h. Actin was used as a loading control. Each blot shown in Figure 4.2A is representative of results obtained in triplicate using separate samples. These triplicate results are represented as changes in protein expression in the graphs in Figure 4.2B. (*) indicates statistical significance at $P \leq 0.1$ and (**) indicates statistical significance at $P \leq 0.05$.

4.4 Discussion

The IGFBPs are a complicated group of proteins with a variety of functions both dependent and independent of IGFs. In this study, the effects of magnolol, a compound found in the roots and bark of the magnolia tree *Magnolia officinalis*, have been examined on the expression of IGF-I, IGF-IR and several IGFBPs in LNCaP and PC3 cells *in vitro*. Magnolol exposure resulted in significantly reduced protein expression of IGF-I in both cell lines after 6 h at a concentration of 80 μM. Changes were also observed in IGF-IR and the IGFBPs at this concentration and time point.

IGF-I functions to increase cellular uptake of amino acids and promote carbohydrate metabolism through stimulation of glycogen and protein synthesis (Yu and Rohan 2000). Stimulation of the cell cycle can also result from elevated IGF-I levels as IGF-I can increase the synthesis and stimulation of cyclin D1 (Furlanetto *et al*. 1994; You *et al*. 2002), and IGF-I expression has been associated with increased protein expression of cyclin D1 and CDK4 in MCF-7 cells (Mawson *et al*. 2005). The decrease in IGF-I observed in the present study corresponds to a decrease in cyclin D1 expression in PC3 and DU145 cells exposed to magnolol *in vitro* as observed in Chapter Two of this thesis (McKeown *et al*. 2014). As alterations in the cell cycle were also observed in the aforementioned study (McKeown *et al*. 2014), it is possible that decreased IGF-I protein expression observed in the present study may be involved in the altered cell cycle response to magnolol previously described.

IGF-I function is mediated by IGF-IR. IGF-R is activated *via* phosphorylation and subsequently activates the phosphatidylinositol 3-kinase (PI3K) and mitogen activated

protein kinase (MAPK) pathways (Shukla and Gupta 2009). As IGF-IR protein expression increased by similar amounts in both LNCaP and PC3 cells, achieving statistical significance only in PC3 cells, this would suggest an increase in IGF-I signaling and therefore increased cellular proliferation. Protein expression of the phosphorylated form, p-IGF-IR, was significantly decreased in PC3 cells, while expression was unchanged in LNCaP cells. This suggests that, while the expression of IGF-IR is increasing, perhaps in a compensatory manner in response to decreased IGF-I expression, p-IGF-IR is either decreasing in protein expression or unchanged and, therefore, IGF-I signaling is not increasing. This relationship of decreased IGF-I and either decreased or unchanged p-IGF-IR could be one further explanation of changes to the PI3K and MAPK signaling pathways in response to magnolol previously observed (Chen and Lee 2013; Chuang *et al*. 2011; Hsu *et al*. 2007; Kim *et al*. 2007; Lee *et al*. 2008a).

IGFBP-2 is directly involved in growth stimulation and has been suggested to be implicated with progression from androgen sensitivity to androgen independence (DeGraff *et al*. 2007, 2009; Moore *et al*. 2003). In the present study the protein expression of IGFBP-2 was not significantly altered from control values in either LNCaP or PC3 cells. This was unexpected as decreases in IGF-I have previously been associated with decreased IGFBP-2 protein expression as IGFBP-2 functions to stabilize IGF-I and thereby increase its half-life (Clemmons 1997).

IGFBP-3 protein expression increased in both LNCaP and PC3 cells after 6 h exposure to magnolol *in vitro*. IGFBP-3 is generally associated with the induction of anti-

metastatic functions and acts as a tumor suppressor in mouse models (Mehta *et al*. 2011). This increase in IGFBP-3 expression could be involved in other magnolol-mediated changes to metastasis observed previously (Chuang *et al*. 2011; Hwang and Park 2010; Kim *et al*. 2007).

In the present study the most pronounced increase to protein expression in both LNCaP and PC3 cells occurred in IGFBP-4 although this increase was only statistically significant in LNCaP cells. IGFBP-4 acts by inhibiting IGF-I action and, therefore, inhibiting cellular growth and proliferation, with the main role of IGFBP-4 in normal cells being the protection of cells from overstimulation by IGF (Clemmons 1997). The increased expression of IGFBP-4 observed in the present study provides an additional and novel pathway by which alterations to cellular growth and proliferation previously described may occur (McKeown *et al*. 2014). When increased IGFBP-4 protein expression is considered in conjunction with decreased IGF-I protein expression the net result is a lowered availability of IGF-I to the cell. This IGF-I is involved in activating cellular signaling pathways, particularly the MAPK and PI3K pathways (Krishna and Narang 2008; Pollak 2008). As previously observed, magnolol exposure results in decreased expression of PI3K proteins (McKeown and Hurta 2015). The PI3K pathway is, in part, responsible for control of proteins involved in progression through the cell cycle (Fresno Vara *et al*. 2004; Vivanco and Sawyers 2002). The expression of these cell cycle proteins have previously been shown to decrease in response to magnolol exposure (McKeown *et al*. 2014). The present study therefore contributes important and novel information to our understanding of how magnolol affects human prostate cancer cell *in*

vitro by highlighting some of the earliest alterations occurring in the cellular signaling mechanism. *In vivo* studies have shown that either an increase or decrease in IGFBP-4 expression can delay tumor formation (Durai *et al*. 2006).

IGFBP-5 expression was significantly lower in both LNCaP and PC3 cells exposed to magnolol. Decreases in IGFBP-5 have previously been associated with decreases in anti-apoptotic activity and may be involved in the apoptotic response to magnolol previously described (Cobb *et al*. 2004; Lin *et al*. 2001). Decreases in IGFBP-5 expression have also been associated with inhibition of IGF-I activities involving DNA synthesis and cellular metabolism (Clemmons *et al*. 1997).

The progression from androgen sensitivity to androgen independence may be inhibited by decreased IGFBP-5 expression (Miyake *et al*. 2000). By decreasing IGFBP-5 expression there is a resultant loss of IGF-I bioavailability, which in turn results in decreased progression through the cell cycle (Furlanetto *et al*. 1994). This is supported by *in vivo* models where IGFBP-5 was found to have no effect on apoptosis but functioned in regulating the cell cycle in prostate cancer cells in a rat model (Thomas *et al*. 1998).

Magnolol exposure to decreases IGF-I expression through two mechanisms: protein expression of IGF-I is directly decreased, as well as indirectly affected by the IGFBPs. By decreasing the availability of IGF-I in these two ways the anti-proliferative and anti-apoptotic effects of magnolol may be controlled by this system of IGF-I and IGFBPs interacting both individually, as well as additively or synergistically. Further

research studies examining the effect of magnolol on other aspects of cancer cell progression and control, such as metastasis and apoptosis, are warranted.

CHAPTER FIVE

General Discussion and Future Directions

5.1 General Discussion

The results obtained through the experiments described in this thesis indicate that magnolol affects cellular growth and proliferation linked processes within human prostate cancer cells *in vitro*. As described in Chapters Two, Three and Four, magnolol causes alterations in the expression of key proteins involved in the cell cycle, polyamine proliferation and IGF regulation.

In Chapter Two, magnolol was shown to be cytotoxic to human prostate cancer cells *in vitro*, confirming the findings of Lee *et al*. (2009) and establishing the effective concentration to be used throughout this thesis. It has also been previously established that magnolol is non-toxic to untransformed cells at these concentrations *in vitro*, and is non-toxic with no associated side-effects in mouse and rat models *in vivo* (Ho and Hong 2012; Lee *et al*. 2009; Li *et al*. 2007; Liu *et al*. 2007). These toxicity experiments did not, however, investigate the effects of magnolol on reproductive health. While no negative reproductive side effects have been associated with magnolol, magnolol is a phytoestrogen and other phytoestrogens have been know to cause impaired fertility and other reproductive disorders and justifies further research in this area be undertaken (Mitchell *et al*. 2001). Furthermore, magnolol was shown to alter cell cycle progression by altering cellular expression of the cyclins, CDKs and their inhibitors. In Chapter Three, magnolol was shown to affect the expression of proteins involved in polyamine

biosynthesis and catabolism as well as the expression of proteins involved in various cellular signaling pathways. Finally, in Chapter Four, magnolol was shown to affect the expression of IGF-I and the IGFBPs which help to regulate it.

While each of these chapters focuses on one aspect of cellular growth or proliferation in human prostate cancer cells *in vitro*, it is the cellular signaling pathways described in Chapter Two which unite these chapters into a single, cohesive project. The proteins of the cell cycle are the driving force of proliferation, and it is this set of proteins which the cellular signaling pathways are ultimately affecting. The polyamines themselves are intricately linked to the cell cycle, with polyamine concentrations being highest in cells during the G_1/S-phase transition (Oredsson *et al*. 1984; Wallace *et al*. 2003). While the exact nature of this connection between the cell cycle and increased polyamine concentration is unknown, it is thought that polyamines are necessary for DNA replication to occur and possibly playing a role in cyclin degradation (Oredsson *et al*. 1984; Wallace *et al*. 2003). The PI3K/Akt signaling pathway was the most consistently altered signaling cascade in response to magnolol in the experiments conducted for this thesis. Downstream of PI3K signaling, both ODC and key cell cycle inhibitors such as p21 and p27 are controlled (Flamigni *et al*. 1997; Fresno Vara *et al*. 2004; Rajeeve *et al*. 2013; Vivanco and Sawyers 2002). It has been suggested that it is this relationship by which PI3K activation can so effectively cause cancerous activity and proliferation: by increasing cellular polyamine concentration through increased expression of ODC and by promoting cell cycle progression through decreased expression of cell cycle inhibitors (Rajeeve *et al*. 2013). As described in this thesis, cells

exposed to magnolol show decreased cell cycle activity, increased expression of p27 and decreased expression of ODC which is consistent with an inhibition of the relationship described above. This thesis is unique in linking alterations to IGF-I and its associated binding proteins, the proteins of polyamine biosynthesis and catabolism and the proteins involved in the cell cycle together through the cellular signaling pathways in the response of human prostate cancer cells to magnolol exposure *in vitro*. All of these processes play an important role in human prostate cancer and are affected by magnolol exposure as is shown in this thesis for the first time.

Cell cycle proteins are also among the downstream targets of the MAPK pathways, the NFκB pathway and the AP-1 pathway. These cellular signaling pathways are further interconnected, with Akt activating the NFκB pathway through inhibition of IκBα, and the NFκB and JNK pathways being major activators of c-jun which in turn activates the AP-1 pathway (Fujioka *et al*. 2004; Vivanco and Sawyers 2002; Wagner and Nebreda 2009). The alterations in protein expression in each of these pathways observed in response to magnolol exposure in this thesis could have led to cell cycle alterations. Upstream of these signaling pathways is IGF-1. While the IGFBPs regulate IGF-I, IGF-I signaling leads to the downstream activation of the PI3K, MAPK, NFκB and AP-1 signaling pathways which ultimately result in changes of expression in proteins involved in the cell cycle as described above (Pollak 2004).

When the data described within this thesis are considered as a whole, they describe magnolol as a compound capable of affecting a wide range of cellular growth and proliferation and linked activities. Magnolol has been shown to cause similar effects

in a variety of cell lines, lending credence to the results found here, as well as strengthening the support for magnolol having an anti-cancer effect (Chen *et al*. 2009; Hsu *et al*. 2007; Lee *et al*. 2008a; Lin *et al*. 2002).

These studies were exclusively based on *in vitro* assays. While *in vitro* studies are effective in the early stages of investigating a compound to determine its usefulness and potential benefits, particularly in justifying later *in vivo* studies by showing a compound to be effective in altering protein expression or cellular behavior, it would be preferable to provide some information acquired through *in vivo* experimentation. The limitation with *in vitro* studies lies in that the cells are directly exposed to the compound of interest, in this case magnolol, without first passing through the digestive tract of the animal nor requiring that the compound be transported to them via the circulatory system. This means that *in vitro* studies can be conducted at much higher concentrations for much longer exposures than are feasible or even possible *in vivo*. To counteract this, a concentration was chosen which should be attainable *in vivo* as discussed above. Similarly, due to the broad nature of experiments conducted within this thesis, *in vivo* experiments would prove much more time consuming and would not be feasibly accomplished within a reasonable timeframe.

Each of the pathways described in this thesis affect cellular growth or proliferation through independent mechanisms discussed throughout this thesis, but it is important to note that these systems are interconnected and do not occur in isolation from one another. The processes described within this thesis are happening in conjunction with one another and so it would be inadvisable to consider any one of these mechanisms the

sole reason for the effect magnolol produces in preventing prostate cancer cell growth *in vitro*. Likewise, it is important to consider other potential factors not discussed within this thesis, which may form the basis for future studies regarding the effect of magnolol in human prostate cancer cells *in vitro*. This thesis shows that magnolol can affect growth and proliferation linked activities in human prostate cancer cells *in vitro* through each of these mechanisms, highlighting the importance of magnolol as a potential chemoprotective and chemopreventative compound in human prostate cancer.

5.2 Future Directions

This project focused on the effects of magnolol on cellular growth and proliferation in human prostate cancer cells *in vitro*, while studies by others investigating the effects of magnolol on apoptosis and metastasis have previously been performed (Hwang *et al*. 2010; Lee *et al*. 2009). Certain aspects of these processes remain to be investigated. The specific mechanism by which IGF-I signaling occurs could be elucidated by measuring protein expression of known signaling targets in the presence of magnolol, IGF-I and both magnolol and IGF-I to determine if magnolol can counteract the effect of excess IGF-I and to ensure that the expression of these specific targets of IGF-I are being affected. The cellular polyamine concentrations could also be measured by HPLC, both in the presence and absence of magnolol, to determine to what extent magnolol-induced changes in the biosynthetic and catabolic proteins affect these polyamine concentrations. While it has been shown by Hwang *et al*. (2010) that magnolol can affect expression of MMPs and inhibit cellular invasion, other proteins involved in cellular adhesion could be examined in more detail (as is currently being done in our lab).

Beyond this, other hallmarks of cancer as described by Hanahan and Weinberg (2011) remain to be investigated, such as the effects of magnolol on angiogenesis. More broadly, the mechanisms by which magnolol interacts with cells to induce the observed effects could be determined, not only to gain insight on how magnolol behaves at the molecular level and induces such cellular responses as those observed, but also to help understand how similar compounds work and to more effectively develop compounds with stronger effects.

It also remains to expand this research from *in vitro* assays to studies involving *in vivo* models of human prostate cancer such as TRAMP mice. This would serve to determine if the concentrations used in this thesis are attainable *in vivo* in practice, as well as determining if magnolol shows similar inhibition of cancer cell growth and proliferation *in vivo*. Further toxicity studies, including the effects of long term magnolol exposure on reproductive health, need also be undertaken. Similar *in vivo* studies have been performed on nude mice implanted with human colon cancer cells, showing that these cells will increase p21 expression and undergo apoptosis (Lin *et al*. 2002). The previous *in vivo* studies, combined with the *in vitro* studies described in this thesis and elsewhere, suggest that magnolol will perform similarly in preventing cellular growth and proliferation *in vivo* and warrant further investigation.

Two compounds closely related to magnolol, honokiol and obovatol, have also been subject to scientific interest for their potential anti-cancer activity. Both honokiol and obovatol have been previously shown to inhibit cell cycle progression in human prostate cancer cells (Hahm *et al*. 2008; Hahm and Singh 2007; Lee *et al*. 2008b). At the time of writing, no papers are known to have been published investigating the effect of either honokiol or obovatol on IGF and the IGFBPs, nor on polyamine proliferation. It would also be of interest to study magnolol, honokiol and obovatol in combination as these compounds exist together in nature. It is possible that cellular responses to these compounds observed in isolation are not representative of the full potential of the compounds produced by *Magnolia officinalis* as these compounds may work

synergistically in nature. These studies would aid in the investigation of this family of compounds and help to characterize their effect on cellular growth and proliferation.

5.3 Summation

In summary, magnolol can affect the behavior of human prostate cancer cells *in vitro*. The studies described within this thesis show that magnolol has anti-cancer activity as well as chemoprotective and chemopreventative effects. These effects include alterations of the cell cycle, as well as changes in expression of proteins involved in the cell cycle, polyamine proliferation and regulation of IGF-I. As these effects are clearly demonstrated, and similar effects can be found in the literature, further studies into the effects of magnolol on prostate cancer cells, particularly *in vivo*, are warranted.

This work shows, for the first time, that magnolol affects expression of proteins involved in polyamine biosynthesis and catabolism. It is also uniquely shown that magnolol affects expression of cell cycle proteins and IGF-I in human prostate cancer cells *in vitro*. These results are significant because they highlight magnolol as a promising anti-cancer compound and justify further research in *in vivo* models of prostate cancer for the further development of magnolol as a chemopreventative and chemoprotective drug.

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APPENDIX A

American Cranberry (*Vaccinium macrocarpon*) Extract Affects Expression of Insulin-Like Growth Factor-I and Insulin-Like Growth Factor Binding Proteins in Human Prostate Cancer Cells *In Vitro*

A.1 Abstract

American cranberry (*Vaccinium macrocarpon*) extract has previously been shown to affect cell cycle, apoptosis and proliferative ability in prostate cancer cells *in vitro*. The insulin-like growth factors (IGFs) and their modulators, specifically the insulin-like growth factor binding proteins (IGFBPs) may play roles in these events. The present study tested IGF-I and IGFBPs expression in DU145 and PC3 prostate cancer cells after exposure to 50 μg/ml or 100 μg/ml of whole cranberry extract (WCE) for 24 h *in vitro*. Western blot analysis was used to show that protein expression levels of IGF-I, IGFBP-2 and IGFBP-5 decrease whereas IGFBP-4 protein expression levels increase and IGFBP-3 protein expression levels were not significantly altered after exposure to WCE. Alteration in expression of IGF-I and the IGFBPs may be physiologically important as these proteins have been linked to changes in the cell cycle, apoptosis and matrix metalloproteinase activity. The changes in IGF-I and IGFBP expression observed in this study also indicate decreased IGF-I activity which suggests an inhibition of cellular growth, induction of apoptosis and anti-metastatic activity *in vitro*. These WCE mediated changes in IGF-I and IGFBP expression are novel and further indicate the potential chemoprotective and chemopreventative actions of cranberry *in vitro*.

A.2 Introduction

Cancer is the second leading cause of death worldwide, with prostate cancer being the second most commonly diagnosed form in men (ACS 2011). As a result, many patients seek to manage this disease through life style and dietary changes (Bemis *et al*. 2006).

The American Cranberry (*Vaccinium macrocarpon*) is a small plant typical of peat bogs in cool temperate regions of eastern North America (Stewart and Nilsen 1995). The fruit of *V. macrocarpon* has been shown to contain biologically active phytochemicals such as anthocyanins, flavonols and proanthocyanidins (Déziel *et al*. 2012; MacLean *et al*. 2011). These compounds have previously been shown to affect cell cycle, apoptosis and proliferative ability *in vitro* in prostate cancer cells (Déziel *et al*. 2010, 2012; MacLean *et al*. 2007, 2011).

One promising mechanistic avenue for the loss of anti-apoptotic and proliferative ability due to exposure to whole cranberry extract previously discussed by Déziel *et al*. (2010, 2012) and MacLean *et al*. (2007, 2011) are the insulin-like growth factors (IGFs) and their modulators, specifically the insulin-like growth factor binding proteins (IGFBPs). The IGFs are responsible for increased cellular growth by increasing metabolic activity and increased mitogenic activity, specifically through anti-apoptotic activity and stimulating the expression of cyclin D1 to progress the cell cycle (Furlanetto *et al*. 1994; Yu and Rohan 2000). These IGFs bind to IGF-receptors (IGF-Rs) on the cell membrane thereby initiating a cascade of reactions to induce the previously mentioned effects. The IGFBPs act by regulating IGF action, specifically through increasing IGF half-life and by either promoting or inhibiting IGF binding to IGF-Rs (Weinzimer *et al*.

2001). The IGFBPs function by having a greater IGF binding affinity than do the IGF-Rs, thereby blocking IGF and IGF-R interaction and protecting IGFs from proteolysis (Yu and Rohan 2000). The IGFBPs can then functionally remove IGF from the system by remaining bound to it, or increase IGF bioavailability by protecting IGFs from proteolysis and thus retain elevated levels of IGF for the cells (Yu and Rohan 2000). The IGFBPs also exhibit a range of IGF-independent activities, particularly in reducing protein synthesis and inducing apoptosis for increased IGFBP-3 expression and inducing mitosis for increased IGFBP-2 and IGFBP-5 expression (Firth and Baxter 2002). Furthermore, IGFBP activity in cancer cells has been the subject of much interest and atypical expression of these IGFBPs has been associated with dramatic changes in cellular behavior such as IGFBP-2 and IGFBP-5 over-expression correlating with the change from androgen sensitivity to androgen independence in prostate cancer cells (DeGraff *et al*. 2007, 2009; Miyake *et al*. 2000). The present study examines the effect of whole cranberry extract (WCE) on IGF-I and IGFBP protein expression in two prostate cancer cell lines *in vitro*.

Our laboratory has previously examined the effects of WCE on various cellular processes and behaviors in prostate cancer cells *in vitro* including cell cycle (Déziel *et al*. 2012), apoptosis (MacLean *et al*. 2011) and mitogenic activity (Déziel *et al*. 2010; MacLean *et al*. 2007). This study was designed to identify and then characterize a cellular process which could unify the previously observed and elucidated effects of cranberry on prostate cancer cells *in vitro* while additionally describing the novel effects of WCE on

the IGF-IGFBP signaling axis; as such the main focus of this study is specifically the effects of WCE on IGF and the IGFBPs.

A.3 Materials and Methods

All antibodies used in this study were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibodies used included: mouse polyclonal anti-actin, anti-IGFBP-2, and anti-IGFBP-3; rabbit polyclonal anti-IGFBP-4 and anti-IGFBP-5; and goat polyclonal anti-IGF-I. Solvents for HPLC analysis of WCE were purchased from Fisher Scientific (Pittsburgh, PA) and reagent-grade acetone, methanol and ethyl acetate were purchased from Pharmco Products Inc. (Brookfield, CT). Diaion® HP-20 was purchased from Supelco, Inc. (Bellefonte, PA). Cyanidin and quercetin glycoside standards were from Chromadex (Irvine, CA) and procyanidin A2 from Indofine Chemical (Hillsborough, NJ). All other chemicals and materials were purchased from Sigma-Aldrich Canada (Oakville, ON) unless otherwise indicated.

A.3.1 Preparation of Cranberry Extract

Cranberry fruit (*Vaccinium macrocarpon*) was harvested in November 2008 at the State Bog in Wareham, Massachusetts. The fruit was flash-frozen in liquid nitrogen and stored at -20 ^{0}C. until use. Whole cranberry extract (WCE) was prepared as described previously (Déziel *et al*. 2012). Briefly, 1 kg of fruit was extracted several times with 300 ml aliquots of 40/40/19/1 methanol/acetone/water/formic acid at room temperature each time pulsing with a Waring blender for 5 min, and filtering after 30 min, and collecting the filtrate. This was repeated with the pulp until most of the color was gone. The combined filtrates were concentrated *in vacuo* and then freeze-dried. Free sugars were removed by applying the sample to a Diaion HP-20 column and washing several times

with distilled water. Elution with methanol followed by acetone, evaporation and freeze-drying of combined extracts produced 10.5 g. of concentrated whole cranberry extract (WCE).

The WCE was analyzed with a Waters HPLC chromatography system equipped with two pumps, an ultraviolet-visible photodiode array detector and Millennium software, using a Waters (Milford, MA) Symmetry C18 reversed phase column (4.6 X 250 mm) and gradient elution program employing solvent A (4% aqueous acetic acid) and solvent B (4% acetic acid in methanol) at a flow rate of 0.8 ml/min as previously described (Déziel *et al*. 2012). The program consisted of: gradient elution from 99% solvent A over 0-30 min, a gradient to 70% solvent A at 70 min. and gradient to 100% solvent B at 90 min. Anthocyannin glycosides are detected at 520 nm, flavonol glycosides at 355 nm and proanthocyanidins at 280 nm. Content of proanthocyanidins was determined gravimetrically, by fractionation of the extract on Sephadex LH-20 to isolate the proanthocyanidins as previously described (MacLean *et al*. 2011). Proanthocyanidins were characterized by MALDI-TOF MS analysis to determine size and distribution of epicatechin oligomers as previously described (MacLean *et al*. 2011; Neto *et al*. 2006).

A.3.2 Cell Culture and Treatment with Whole Cranberry Extract

Human DU145 prostate adenocarcinoma cells (ATCC, Manasses, VA) and human PC3 prostate adenocarcinoma cells (ATCC) were cultured on 100 mm plastic tissue-culture dishes (Falcon, Mississauga, ON) in α-MEM (Gibco, Burlington, ON) or RPMI (Gibco)

respectively, supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Gibco), and were incubated at 37 ^{0}C in 5% CO_2. At 70% confluence the media was replaced with serum free α-MEM or RPMI, for DU145 cells and PC3 cells, respectively, supplemented with 5 μg/ml of transferrin and 2.5 μg/ml of insulin and incubated for 24 h at 37 ^{0}C in 5% CO_2. After incubation, cells were exposed to either 50 μg/ml or 100 μg/ml concentrations of WCE, dissolved in dimethyl sulfoxide (DMSO), for 24 h. Control cells received only DMSO. After treatment, cells were removed by trypsin diluted in phosphate buffered saline (PBS) and re-suspended in either α-MEM or RPMI, for DU145 cells and PC3 cells, respectively, with 10% FBS and centrifuged for 5 min at 500x G. The media was then aspirated off leaving only a cell pellet, and the cells re-suspended and washed in cold PBS. Cells were again centrifuged for 5 min at 500x G. After centrifugation the PBS was aspirated off and the cell pellet was stored at -80 ^{0}C. until further analysis.

A.3.3 Immunoblot Analysis

Cell pellets were resuspended in 100 μl of 10 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM PMSF, and briefly sonicated. Cell lysates were then centrifuged at 9300x G for 20 min at 4 ^{0}C. The supernatant was removed from the pellet and evaluated for protein content. Equal amounts of protein from this extract were mixed in a 3:1 ratio with standard Laemmli buffer consisting of 50 mM Tris-HCl (pH 6.8), 10% SDS, 0.1% bromophenol blue, 10% glycerol and 100 mM β-mercaptoethanol and boiled for 3 min. Electrophoresis through 10% SDS-PAGE gels was used to resolve proteins which were

then transferred onto nitrocellulose membranes (Bio-Rad, Mississauga, ON) by electroblotting. Membranes were then incubated in the presence of a 1% BSA (w/v) TBS-Tween (0.05% v/v) solution overnight at 4 ^{0}C. Primary antibodies diluted to 1:200 (v/v) were then applied to membranes and incubated for 1 h at room temperature. After incubation, the membranes were washed 3 times with TBS-Tween (0.05% v/v) for a total of 30 min then incubated with alkaline phosphatase (AP)-conjugated secondary antibodies (1:1000) for 1.25 h at room temperature. After incubation the membranes were again washed 3 times with TBS-Tween (0.05%) for a total of 30 min and rinsed quickly with distilled water, then exposed to SigmaFast BCIP-NBT tablets dissolved in distilled water to visualize protein expression levels. Western blots were then photographed using Infinity Capture Software (Lumenera Corp., Ottawa, ON) and densitometry was performed with ImageJ Software (National Institute of Health, Bethesda, MD).

A.3.4 Statistical Analysis

Statistical analysis was conducted using Graphpad Prism 4.03 for Windows (Graphpad Software, Inc. San Diego, CA). Results of immunoblot analysis were compared using a one way ANOVA with a Tukey's Posthoc test and results were considered statistically significant at $P \leq 0.05$.

A.4 Results

A.4.1 Analysis of Whole Cranberry Extract

The WCE used in this study was analyzed concurrently with those used in previous studies, as such characterization of the WCE has been described previously (Déziel *et al*. 2012; MacLean *et al*. 2011; Neto *et al*. 2006). The HPLC profile of this sample has also been previously reported (Déziel *et al*. 2012). Briefly, the major anthocyanin constituents identified in the WCE are cyanidin-3-galactoside, cyanidin-3-arabinoside, peonidin-3-galactoside and peonidin-3-arabinoside. The WCE contained 10.3% anthocyanin by weight. The flavonol glycosides identified were quercetin-3-*O*-galactoside (major glycoside present), myricetin-3-galactoside, myricetin-3-arabinoside, quercetin-3-xyloside, quercetin-3-arabinoside and quercetin-3-rhamnoside. The total flavonol glycoside content of the WCE was 9.9%. Peaks consistent with proanthocyanidins and p-coumaric acid derivatives are also present. The total proanthocyanidin (PAC) content of the extract was 41% by weight. Proanthocyanidins were verified by characteristic peak elution patterns at 279.1 nm (which is the absorbance maximum for epicatechin-based proanthocyanidins). MALDI-TOF MS analysis found the PAC fraction contained proanthocyanidin oligomers ranging in size from two to twelve epicatechin units, primarily dimers (including procyanidin A2), trimers and tetramers with at least one A-type linkage between units, as previously reported (MacLean *et al*. 2011).

A.4.2 Effects of Whole Cranberry Extract on Expression of IGF-I and IGFBPs

As shown in Figure A.1, IGF-I expression was significantly reduced in PC3 and in DU145 human prostate cancer cells after 24 h of exposure to 100 μg/ml WCE. This WCE exposure resulted in a reduction to 50% of control IGF-I at 100 μg/ml in PC3 cells, with a reduction to 67% of control observed at 50 μg/ml in PC3 cells. Similarly, in DU145 cells, WCE exposure resulted in a significant IGF-I reduction to 33% and 22% of control at 50 and 100 μg/ml respectively. To determine if changes in IGF-I expression in response to WCE were affected by changes in the IGFBPs, protein expression of IGFBP-2, IGFBP-3, IGFBP-4 and IGFBP-5 were also examined. Of the IGFBPs examined, significant changes to protein expression were observed in IGFBP-2, IGFBP-4 and IGFBP-5. In DU145 cells, the expression of IGFBP-2 was significantly reduced to 75% and 47% of control after 24 h of exposure to 50 and 100 μg/ml WCE, as shown in Figure A.2. This suppression was also observed to a lesser extent in PC3 cells, with no significant change at 50 μg/ml and a reduction to 77% of control at 100 μg/ml WCE (Figure A.3). IGFBP-3 expression was not significantly altered at any exposure concentration after 24 h, however, a trend towards decreased expression in DU145 cells (Figure A.2) and increased expression in PC3 cells was evident (Figure A.3). IGFBP-4 expression did not change significantly in DU145 cells (Figure A.2), but a significant increase to 140% of control was observed at 50 μg/ml after 24 h in PC3 cells, while no significant change observed in response to treatment with 100 μg/ml WCE after 24 h exposure as illustrated in Figure A.3. As shown in Figure A.2 and Figure A.3, respectively, expression of IGFBP-5 decreased significantly in both DU145 cells and PC3 cells after 24 h exposure

to 100 μg/ml WCE to 73% and 66% of control respectively. PC3 cells also exhibited a significant decrease to 72% of control after exposure to 50 μg/ml WCE for 24 h (Figure A.3).

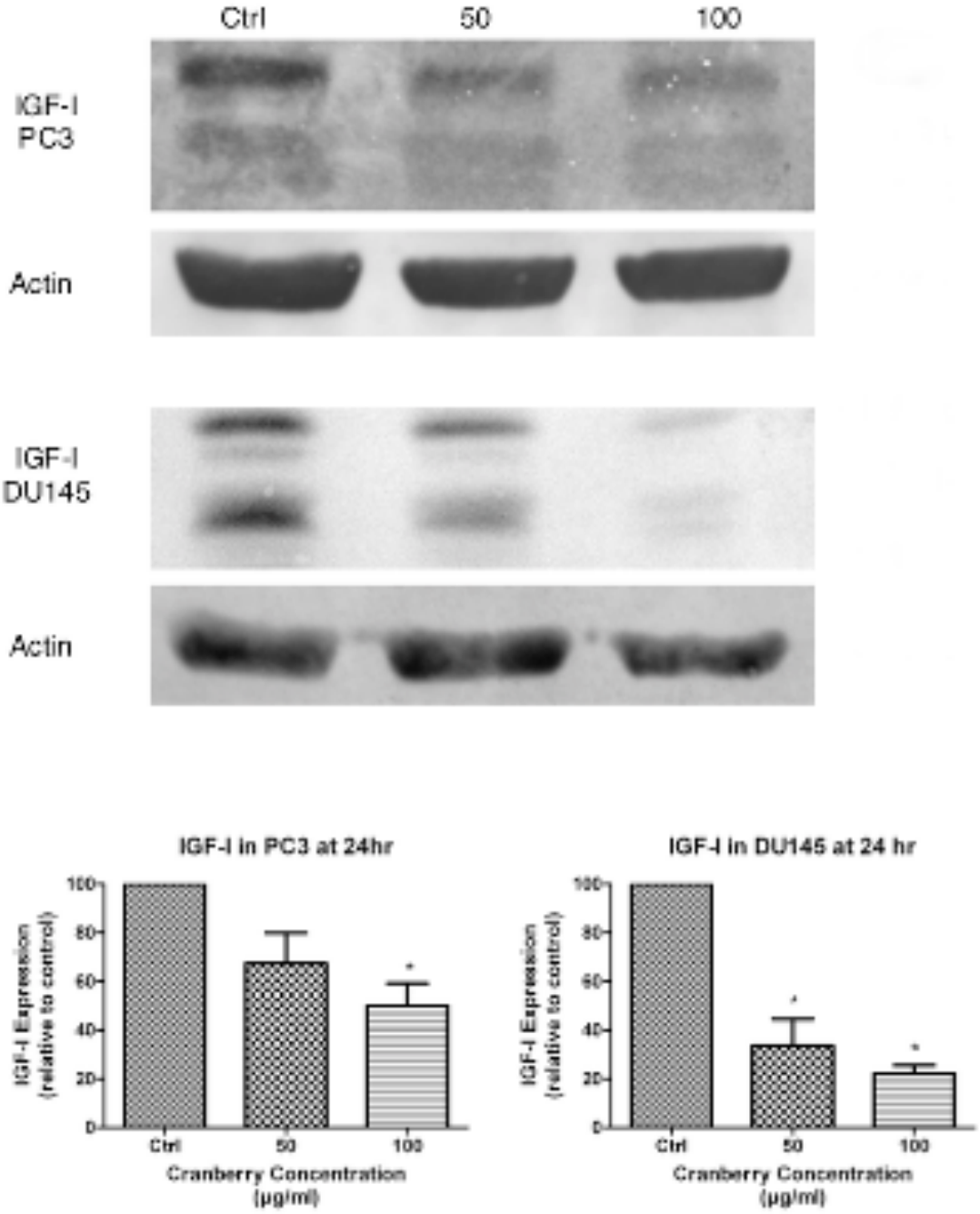

Figure A.1: The effect of WCE on expression of IGF-I in PC3 and DU145 cells. Cells were treated with either DMSO (Ctrl), 50 or 100 µg/ml WCE in DMSO for 24 h. Representative IGF-I expression levels are shown with actin used as a loading control. Experiments were performed with at least three replications using separate samples. (*) indicates statistical significance at $P \leq 0.05$.

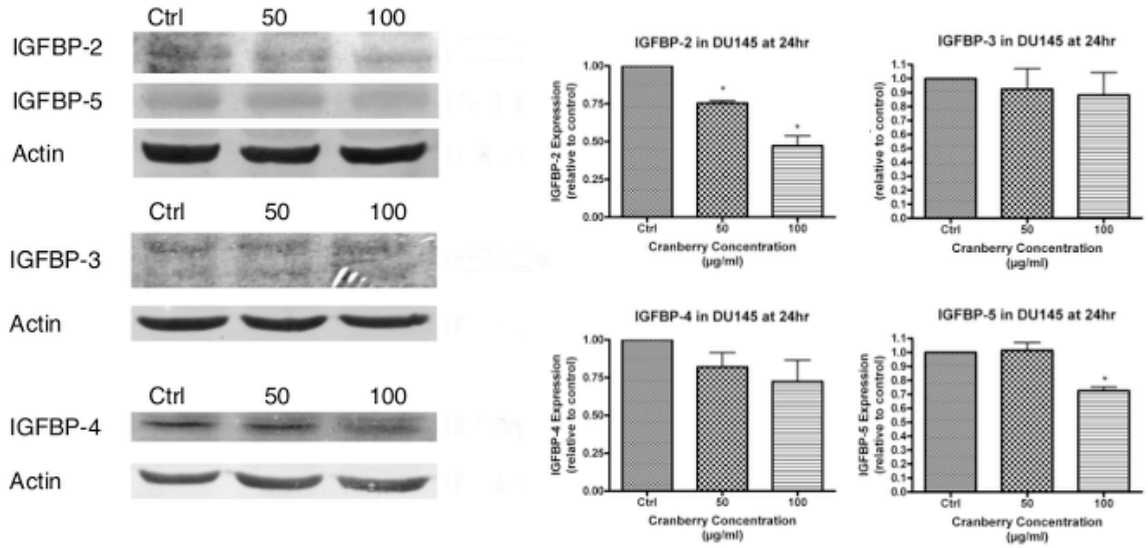

Figure A.2: The effect of WCE on expression of IGFBPs in DU145 cells. DU145 cells were treated with either DMSO (Ctrl), 50 or 100 μg/ml WCE in DMSO for 24 h. Representative expression levels of IGFBP-2, -3, -4 and -5 are shown with actin used as a loading control. Experiments were performed with at least three replications using separate samples. (*) indicates statistical significance at $P \leq 0.05$.

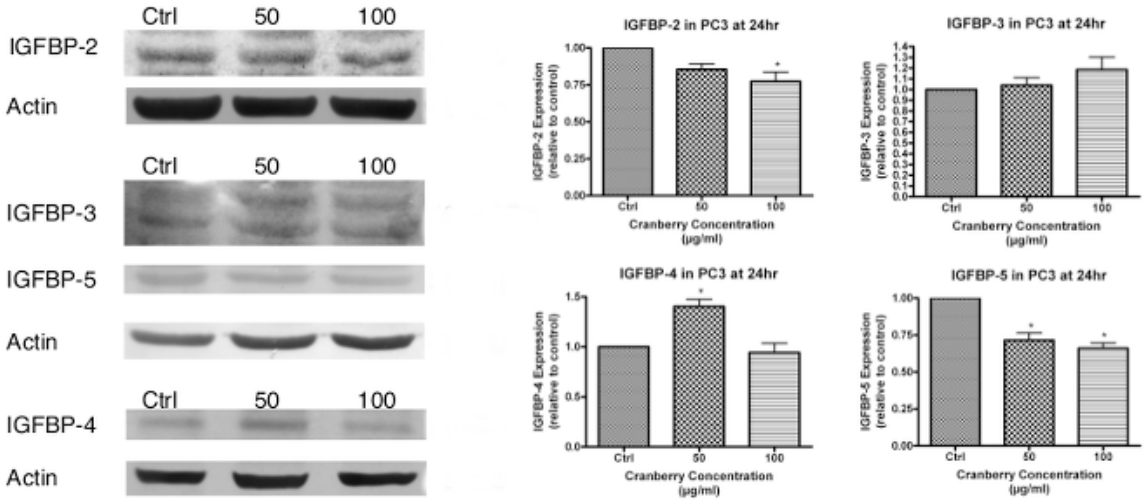

Figure A.3: The effect of WCE on expression of IGFBPs in PC3 cells. PC3 cells were treated with either DMSO (Ctrl), 50 or 100 µg/ml WCE in DMSO for 24 h. Representative expression levels of IGFBP-2, -3, -4 and -5 are shown with actin used as a loading control. Experiments were performed with at least three replications using separate samples. (*) indicates statistical significance at $P \leq 0.05$.

A.5 Discussion

The IGFBPs are a complicated group of proteins with a variety of functions both dependent and independent of IGFs. In this study the effects of WCE from the American cranberry (*Vaccinium macrocarpon*) on expression of IGF-I and several IGFBPs in DU145 and PC3 cells *in vitro* has been examined. WCE was observed to induce significant changes in IGF-I and several IGFBPs after 24 h. The most interesting changes occurred in the expression of IGF-I, IGFBP-2 and IGFBP-5 which were significantly reduced in DU145 cells and PC3 cells.

IGF-I is a growth factor which is normally expressed at peak levels during puberty, at which point expression gradually decreases with age (Furlanetto *et al*. 1994). The function of IGF-I is to increase cellular uptake of amino acids and promote carbohydrate metabolism through stimulation of glycogen and protein synthesis (Yu and Rohan 2000). IGF-I can also act as a potent mitogen by increasing DNA synthesis and stimulation of cyclin D1, causing the cell cycle to progress from G_1 to S-phase, and suppression of cyclin D1 can abolish the mitogenic effect of IGF-I (Furlanetto *et al*. 1994; Mawson *et al*. 2005). The decrease in IGF-I observed in the present study corresponds to a decrease in cyclin D1 expression in DU145 cells exposed to WCE *in vitro* reported by Déziel *et al*. (2012). IGF-I expression is associated with increased expression of cyclin D1 and CDK4 in MCF-7 cells (You *et al*. 2002), both of which are observed to decrease in DU145 cells in response to WCE by Déziel *et al*. (2012). Déziel *et al*. (2012) also observed that cell cycle arrest occurred in the G_1 phase; cyclin D1 and CDK4 are two cell cycle proteins responsible for the induction of S-phase in the cell

cycle. This suggests that decreased IGF expression, as observed in the present study, may be in part responsible for arrest of the cell cycle in DU145 cells in response to cranberry.

Apoptosis is also inhibited by IGF-I through increased expression of Bcl proteins which acts to suppress Bax (Yu and Rohan 2000). The reduced expression of IGF-I observed in this study supports previous work done on the apoptotic effect of WCE. MacLean *et al*. (2011) found that DU145 cells exposed to 10 and 100 μg/ml WCE for 6 and 24 h exhibited increased expression of Bax. As the ratio of Bax to Bcl is used to determine if apoptosis will occur, MacLean *et al*. (2011) concluded that the balance of these two protein may favor apoptosis after WCE treatment. The decrease in IGF-I expression observed in the present study may be one factor contributing to the previously observed increase in Bax expression.

IGF-I has previously been found to increase MMP-2 and MMP-9 activity in DU145 human prostate cancer cells (Saikali *et al*. 2008). Similarly to the WCE associated cell cycle arrest and anti-apoptotic effects, previous studies have associated exposure to WCE (MacLean *et al*. 2007) and proanthocyanidins derived from whole cranberry (Déziel *et al*. 2010) to the inhibition of MMP-2 and MMP-9 activity. Extract fractions from the lowbush blueberry (*Vaccinium angustifolium*) containing anthocyanins and proanthocyanidins have also previously been found to inhibit MMP-2 and MMP-9 in DU145 cells *in vitro* (Matchett *et al*. 2005). Matrix metalloproteinases (MMPs) allow cancer cells to penetrate the vasculature and spread to other parts of the body by degrading the extracellular matrix (Nemeth *et al*. 2002). Déziel *et al*. (2010) observed a significant reduction in MMP-2 and MMP-9 activity in response to cranberry PACs

exposure at 25 µg/ml for 3 h. As the WCE used in the present study contained 41% PACs by weight, it is suggested that decreased IGF-I expression and decreased MMP activity may be associated with the PACs present in the WCE used in this study. Furthermore, MMP activity as well as expression of proteins modulating MMP activity such as tissue inhibitor of metalloproteinase (TIMP), reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) and extracellular matrix metalloproteinase inducer (EMMPRIN) have also been shown to be affected by WCE in 0.1 mg/ml concentrations for a 6 h exposure (MacLean *et al*. 2007). The decrease in IGF-I observed in the present study could be one factor accounting for the changes in MMP expression in addition to WCE's previously reported effects.

IGFBP-2 has previously been observed to stimulate growth in cancerous prostate cells (DeGraff *et al*. 2007; Moore *et al*. 2003), and has been suggested as a potential biomarker of prostate cancer progression from androgen sensitivity to androgen independence (DeGraff *et al*. 2007, 2009). IGFBP-2 is known to be lysed by androgens (DeGraff *et al*. 2007, 2009; Moore *et al*. 2003) and castration in mice has been shown to increase IGFBP-2 expression (DeGraff *et al*. 2009). The proposed mechanism is that castration causes an increase in IGFBP-2 due to reduced proteolysis by androgens, which in turn serves to increase IGF activity (DeGraff *et al*. 2009).

A reduction in IGFBP-2 has previously been associated with a decrease in IGF activity and a reduction in IGF-I half-life due to the loss of stability occurring when IGF-I and IGFBP-2 are bound (Clemmons 1997). A loss of IGF-I due to increased proteolysis, caused by decreased IGFPB-2 expression meaning there is less available IGFBP-2

available to confer stability to IGF-I, can cause a decrease in IGF-I. This decrease in IGFBP-2 corresponds to the decrease in IGF-I found in the present study, and is likely involved in the cell cycle arrest, anti-apoptotic and anti-MMP activity observed in response to WCE and PACs in previous studies (Déziel *et al*. 2010, 2012; MacLean *et al*. 2007, 2011; Neto *et al*. 2006).

A reduction in the IGF-independent activities of IGFBP-2, such as the induction of proliferation and apoptotic resistance (DeGraff *et al*. 2009) is also observed in previous studies on whole cranberry extract through reduced growth and viability of DU145 cells (Déziel *et al*. 2012). It has also been suggested that IGFBP-2 functions differently in DU145 cells and PC3 cells, with IGFBP-2 acting primarily through modulation of IGF activity in PC3 cells but having both IGF-dependent and independent activity in DU145 cells, but the mechanism of this difference between cell lines has not been elucidated (Uzoh *et al*. 2011). This different mode of functionality could explain the discrepancy between results found in DU145 cells and PC3 cells, where a more pronounced IGFBP-2 expression decrease was observed in DU145 cells.

No significant change was observed in IGFBP-3 expression in either DU145 cells or in PC3 cells after 24 h exposure to WCE at either concentration studied. IGFBP-3 is generally associated with the induction of anti-metastatic functions and acts as a tumor suppressor in mouse models (Mehta *et al*. 2011). Studies on grape seed proanthocyanidins in mouse models have shown increased expression of IGFBP-3 and associated suppression of IGF-I leading to anti-cancer effects (Akhtar *et al*. 2009). As such, it was unexpected that no change in IGFBP-3 would occur, but suggests that WCE

acts through expression of proteins other than IGFBP-3. It would be valuable to examine the effects of the various fractions of WCE, such as PACs, to determine if those fractions could induce a change in IGFBP-3.

IGFBP-4 acts by inhibiting IGF-I action and therefore inhibiting cellular growth and proliferation (Clemmons 1997). As mentioned above, a decrease in IGF-I activity can lead to cell cycle arrest, anti-apoptotic and anti-MMP activity as observed in response to WCE and PACs in previous studies (Déziel *et al*. 2010, 2012; MacLean *et al*. 2007, 2011; Neto *et al*. 2006). Clemmons (1997) mentions that the main role of IGFBP-4 is in protecting cells from overstimulation by IGFs and that IGFBP-4 appears to be the only IGFBP to function solely in inhibiting IGF action. Previous studies have found that while over expression of IGFBP-4 does decrease growth of prostate cancer cells by inhibiting IGF action, a reduction in IGFBP-4 expression was associated with the induction of apoptosis as well (Durai *et al*. 2006). *In vivo* studies, in a mouse model, have shown that both an increase and a decrease in IGFBP-4 expression can delay tumor formation (Durai *et al*. 2006).

An increase in IGFBP-4 has previously been associated with lowered expression of IGFBP-2 and increased expression of IGFBP-3 (Drivdahl *et al*. 2001). While a decrease in IGFBP-2 was observed in the present study, there was no significant change in IGFBP-3. IGFBP-4 expression was only significantly increased in PC3 cells at 50 µg/ml WCE after 24 h, but not at the higher concentration.

IGFBP-5 expression was significantly lower in both DU145 cells and PC3 cells at 24 h in the presence of 100 µg/ml WCE in the present study. This decrease in IGFBP-5

expression may lead to a decrease in anti-apoptotic activity as described by Cobb *et al*. (2004) who observed that elevated levels of IGFBP-5 were associated with decreased apoptosis; this decrease in apoptosis occurred due to an inhibition of the intrinsic pathway of apoptosis and is independent of IGF. Inhibition of IGFBP-5 mRNA has been shown to inhibit cell proliferation, by causing cell cycle arrest, but did not affect apoptosis (Miyake *et al*. 2000). Likewise, decreases in IGFBP-5 expression have been associated with inhibition of IGF-I activities involving DNA synthesis and cellular metabolism (Clemmons 1997).

This suggests that, like IGFBP-2, decreasing IGFBP-5 expression may inhibit progression from androgen sensitivity to androgen independence as up regulation of IGFBP-5 in prostate cancer cells after castration helps to promote the proliferative effects of IGF-I (Miyake *et al*. 2000). As with IGFBP-2, decreasing IGFBP-5 expression will result in a loss of bioavailability of IGF-I which in turn will result in a decrease in cyclin D1 expression and therefore inhibit progression through the cell cycle (Furlanetto *et al*. 1994). This is supported by *in vivo* models where IGFBP-5 was found to have no effect on apoptosis but functioned in regulating the cell cycle in prostate cancer cells in a rat model (Thomas *et al*. 1998). The IGF-independent activity discussed by Cobb *et al*. (2004) would also suggest that the decrease in IGFBP-5 expression will lead to an increase in apoptosis observed by MacLean *et al*. (2011).

WCE also decreases IGF-I expression through two approaches: directly influencing IGF-I expression and indirectly influencing IGF-I expression through alterations to IGFBP expression. This dual approach to decreasing IGF-I expression could

cause the inhibition of cyclin D1 expression and the induction of Bax expression as observed in previous studies (Déziel *et al*. 2012; MacLean *et al*. 2011) on WCE and further provides support that WCE can cause an arrest of cell cycle and the induction of apoptosis as previously described.

Finally, further research studies examining the effect of enriched fractions of WCE (including PACs, anthocyanins, flavonols, ursolic acid and its esters, etc.) on IGF-I and the IGFBPs to determine what components of WCE are active in affecting IGF and IGFBPs in prostate cancer cells *in vitro* are warranted. Future projects are currently planned to describe the signaling pathways controlling IGF and IGFBP expression.

In conclusion, the IGFBPs are part of a complex group of proteins in the IGF family and are primarily responsible for the regulation of the IGFs. Exposure to WCE can affect IGF and IGFBP expression in DU145 and PC3 prostate cancer cells *in vitro* causing a decrease in IGF-I, IGFBP-2 and IGFBP-5 expression, and an increase in IGFBP-4 expression. These changes in expression of the IGFBPs may be responsible for inhibition of IGF activity, causing a reduction in the proliferative and anti-apoptotic effects of the IGFs. It has also been suggested that an increase in IGFBP-2 and IGFBP-5 expression is correlated with the transition from androgen sensitivity to androgen independence in prostate cancer cells. In demonstrating that IGFBP-2 and IGFBP-5 expression is decreased by exposure to WCE, it is possible that this may hold future potential as a method to block expression of these two proteins and thereby delay prostate cancer progression in this manner.